In Vitro Modelling of Cellular Processes in OM-BMDM Studies in Junbo Mice Reveal Defects in HIF and TGF- Signalling

Thesis

Abstract

***In vitro* modelling of cellular processes in OM- BMDM studies in *Junbo* mice reveal defects in HIF and TGF-β signalling**

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Chronic otitis media (OM) is a common cause of deafness in children. Two novel murine models of chronic OM, *Junbo* and *Jeff,* generated by chemical mutagenesis with ethyl nitrosurea (ENU), have been developed at MRC Harwell. *Junbo* heterozygote mice (*Jbo/+*) mice have a mutation in transcription factor *Evi-1* and *Jeff* heterozygote mice *(Jf/+*) mice have a mutation in gene for Fbxo protein *Fbxo11*. Pathologic hypoxia has been characterized as a common feature of the inflamed middle ear in both models. HIF-VEGF pathways dysregulation has been indicated in both models previously. However, the mechanisms involved in pathogenesis of OM in these two models are yet to be elucidated.

In this thesis I have described an *in-vitro* model system to study cellular processes in OM to study and identify pathways involved in OM pathogenesis. This model system involves culturing BMDM and exposing them to various treatments under standard defined conditions.

Results from this thesis reveal that Evi-1 is expressed at comparable levels in both WT and *Jbo/+* genotypes and that *Evi-1*^{A2288T} mutation is a loss of function mutation which results in dysregulated expression of Smad responsive and hypoxia responsive genes such as *Vegf* and *Glut-1* under prolonged hypoxic conditions. Although *Jbo/+* BMDM are hyper-responsive to LPS in normoxia, as was indicated by higher *Vegf* and *Il-6*

levels, macrophage phagocytic function is potentially attenuated in *Jbo/+* under hypoxic conditions as was indicated by lower levels of *Tnf-α* and *Il-1β* in *Jbo/+* BMDM in LPS and hypoxia combination treatment. A perturbed resolution of *Il-6* and *Il-1β* in hypoxia was also observed.

Studies performed in this thesis revealed dysregulation in TGF-β pathway in both *Jbo/+* and *Jf/+* BMDM in normoxia which suggests a common role of TGF-β signalling in OM pathogenesis. Differential expression of cytokines was observed in *Jbo/+* BMDM after prolonged treatment with TGF-β and hypoxia indicating a pro-angiogenic and pro-inflammatory phenotype. Levels of pro-inflammatory cytokines such as Il-1β, C5a, Il-17, Il-23, and Ccl5 were higher in *Jbo/+* BMDM and levels of anti-inflammatory cytokines such as Il-1ra, Il-10, and Il-4 were lower in *Jbo/+* BMDM after prolonged hypoxia and TGF-β treatment which further validates the role of dysregulated HIF and TGF-β signalling pathways in OM in *Jbo/+* mice. The differential cytokine expression observed in *Jbo/+* BMDM is favourable for Th17 cell differentiation and may indicate a role of cross-talk of innate immunity and cell mediated immunity in OM chronicity in hypoxia.

To conclude, HIF signalling and TGF-β signalling in *Jbo/+* BMDM is dysregulated. Pro-angiogenic function, impaired resolution of inflammation and predisposition to Th17 cell differentiation by *Jbo/+* BMDM under hypoxia contribute to the prolonged and chronic inflammation in *Jbo/+*mice and may provide some evidence of pathways that could be affected in OM patients.

Keywords- HIF- hypoxia inducible factor; TGF-β- Transforming growth factor β; BMDM- Bone marrow derived macrophages, OM (Otitis media), mouse models, hypoxia, inflammation.

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Abbreviations

293T cells	Human Embryonic Kidney 293 cells
Abca1	ATP-binding cassette transporter
ABR	Auditory brainstem response
ACAMPs	Apoptotic cell associated molecular patterns
Akt	v-Akt murine thymoma viral oncogene homolog
AML	Acute myeloid leukemia
AOM	Acute otitis media
Ap-1	Activator protein-1
BM	Bone marrow
BMDNLs	Bone marrow derived neutrophils
C5a	Complement component 5 anaphylatoxin
CAMs	cell adhesion molecules
CBP	CREB binding protein
CDC	Centre of disease control
cDNA	complementary DNA
CML	chronic myeloid leukemia
COME	Chronic otitis media
CRS	Chronic rhinosinusitis
CSOM	Chronic otitis media
CtBP	C-terminal binding protein
d	day/s
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
E2F4	E2F transcriptional factor 4
ENU	N-ethyl-N-nitrosourea

ET	Eustachian tube
EUCOMM	European conditional mouse mutagenesis program
Evi-1	Ecotropic viral integration site-1
Eya4	Eyes absent homolog
FACS	Fluorescence-activated cell sorter
Fbxo11	F-box only protein 11
FCS	Fetal calf serum
FIH	Factor inhibiting HIF
FITC	Fluorescein isothiocyanate
Foxp3	forkhead box P3
g	Unit of gravity
g	gram
G-CSF	Granulocyte colony stimulating factor
Glut-1	Glucose transporter-1
GM-CSF	Granulocyte/macrophage colony stimulating factor
GWAS	Genome wide association studies
h	hour/s
HBSS	Hank's Balanced Salt Solution
HIF	Hypoxia inducible factor
IBD	Inflammatory Bowel Disorder
IFNγ	Interferon gamma
IKMC	International knockout mouse consortium
IL-10	Interleukin-10
Il-1ra	Interleukin-1 receptor antagonist
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8

I-tac	Interferon-inducible T-cell alpha chemoattractant
Jbo/+	*Junbo* heterozygote
Jf/+	*Jeff* heterozygote
JNK	c-Jun N-terminal kinase
kD	kilodalton
KI	knockin
KO	knockout
LDL	Low density lipoprotein
Lpr/lpr	homozygous recessive lymphoproliferation mutation
LPS	Lipopolysaccharide
LXR	Liver X receptor
MAPK	Mitogen-activated protein kinase
Mcp-1	Monocyte chemotactic protein-1
M-csf	Macrophage-colony stimulating factor
MDS	Myelodysplastic syndrome
ME	Middle ear
min	minute
Mip-2	Macrophage Inflammatory Protein-2
MMP	Matrix metalloproteinases
MMRs	Macrophage mannose receptor
mol	mole
mTOR	mammalian target of rapamycin
MyD88	Myeloid differentiation primary response protein 88
n	Number in study or group
NF-κB	Nuclear factor-kappa beta
NK	Natural Killer cells
NLs	Neutrophils

NO	Nitric oxide
NT*Hi*	Non-typeable *Haemophilus influenzae*
OM	Otitis media
OME	Otitis media with effusion
Ox-LDL	Oxidized low density lipoprotein
p53	Protein 53
p73	Transformation-related protein 73
Pai	Plasminogen activator inhibitor
PAMPs	Pathogen associated molecular patterns
PAS domain	Per-ARNT-Sim domain
PBS	Phosphate buffered saline
PcG	Polycomb gene complex
PCR	Polymerase chain reaction
PHD	Prolyl hydroxylase domain containing protein
PI	Propidium Iodide
PI3k	Phosphatidylinositol 3-kinase
PIMO	Pimonidazole
PMN	polymorphonuclear leukocytes
PRR	Pathogen recognition receptors
Pten	Phosphatase and tensin homolog
PU.1	PU-box transcription factor
RA	Rheumatoid arthritis
RAOM	Recurrent otitis media
RNS	Reactive nitrogen species
RORγt	Retinoic acid receptor related orphan receptor gamma
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium

RSV	Respiratory syncytial virus
RT-qPCR	Real-time quantitative PCR
Runx1	Runt-related transcription factor 1
RXR	Retinoid X receptor
s	second
SD	Standard deviation
SEM	Standard error of mean
siRNA	Small interfering RNA
SNPs	Single nucleotide polymorphisms
TGF-β	Transforming growth factor β
Th	T helper cell
Th1, Th2	T helper cells 1, T helper cells2
Th17	T helper cells 17
THP-1 cells	Human acute monocytic leukemia cell line
Timp	Tissue inhibitor of metalloproteinases
TLR-2	Toll like receptor-2
TLR-4	Toll like receptor-4
TNF-α	Tumor necrosis factor-α
tPA	tissue plasminogen activator
Tregs	Regulatory T cells
Trem-1	Triggering receptor expressed on myeloid cells 1
uPA	urokinase plasminogen activator
URT	Upper respiratory tract
Vegf	Vascular endothelial growth factor
VHL	Von hippel-lindau gene
WHO	World health organization
wk	week

WT	Wild-type
ZF1, ZF2	Zinc finger domain 1, Zinc finger domain 2

1. Contents

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Chapter 1

2. Introduction

Normal ear anatomy and Otitis media (OM)

Irwin describes the human ear as a "complex series of interlinked structures" involved in hearing (Irwin, 2006). These structures are broadly divided into external ear, middle ear and inner ear. Sound waves are collected by the external ear and pass onto a thin, oval, semi-transparent membrane known as the tympanic membrane. The tympanic membrane is attached to a chain of three small movable bones (ossicles) that transmit sound vibrations to the inner ear (Antonelli, 2004). These three ossicles (malleus, incus and stapes), along with the openings of both the Eustachian tube (ET) and the Mastoid air cell, are present in the tympanic cavity that constitutes the middle ear (Gray, 1918). The tympanic membrane forms the outer border of the middle ear, which is connected to the pharynx and the back of the nose via the ET and the mastoid antrum via the aditus (Gray, 1918; Irwin, 2006). Also present in the middle ear are two tiny muscles – the Stapedius and Tensor tympani, which protect the ears from noise trauma by contracting in response to loud noises (Gray, 1918; Irwin, 2006). The tympanic cavity is defined in Gray's anatomy of the human body as "an irregular lateral compressed air-filled space within the temporal bone". It is lined with respiratory mucus epithelium. The temporal brain lies superior to the middle ear and jugular vein, and inferior to the carotid artery (Gray, 1918; Irwin, 2006).

The main function of the middle ear is to transmit sound vibrations from the air to the inner ear, and the air inside the tympanic cavity is essential for the efficient vibration of the tympanic membrane and ossicles in response to

sound (Gray, 1918; Irwin, 2006). When the middle ear is inflamed, the air in the tympanic cavity is replaced by fluid, which interferes with sound transmission to the inner ear (Antonelli, 2004). This results in conductive hearing impairment, which is often associated with middle ear inflammation or otitis media.

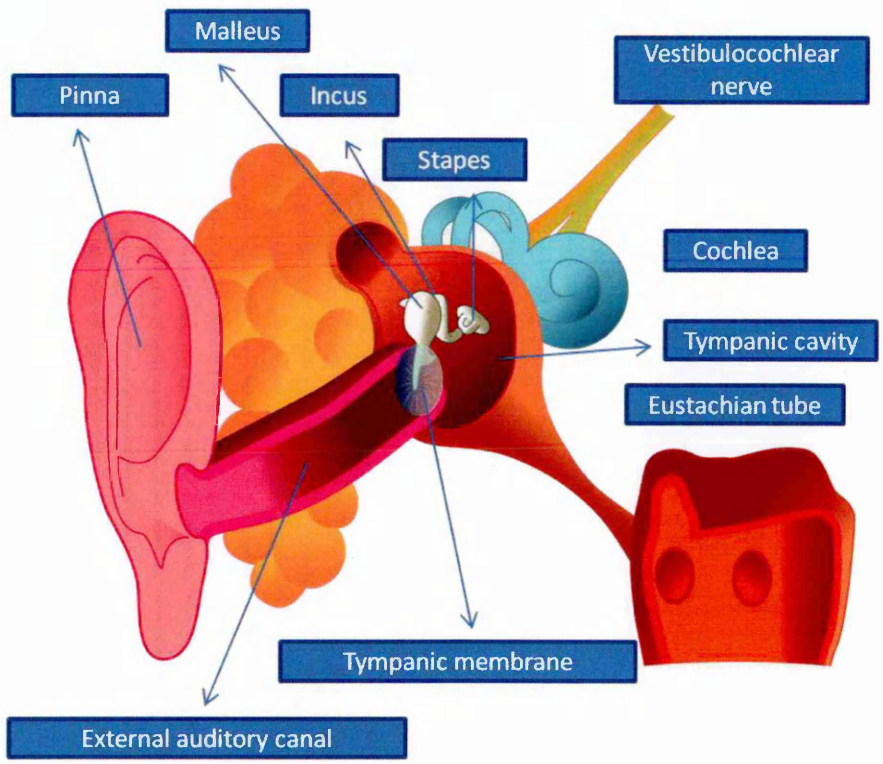


Figure 2.1 Anatomy of the middle ear

This diagram is a graphical representation of the human middle ear. In otitis media, tympanic cavity is filled with fluid which interferes with effective transmission of sound vibration to the inner ear. (Courtesy Dr. Mahmood Bhutta)

1.1.1- Classification of Otitis Media (OM)

Otitis media (OM) is a multi-factorial disease and the most common cause of deafness and surgery in children of developed countries (Clarke & De, 2005; Kubba et al, 2000). The terminology used to describe OM and its sequelae in this thesis is consistent with the current understanding of the disease. OM manifests itself in the following forms in humans:

1.1.1.1- Acute Otitis Media (AOM)

Acute otitis media (AOM) is the most common form of OM and presents itself as an "acute suppurative inflammation" with fever, ear pain and eardrum redness (Kong & Coates, 2009). Bulging of the tympanic membrane is also often observed (Kong & Coates, 2009). AOM is frequently elicited by bacterial or viral infections. Most children have had at least one episode of AOM by the age of three. AOM is the most common childhood bacterial infection (Teele et al, 1989a; Teele et al, 1989b). Although mostly associated with bacterial infection, AOM has also been associated with viral infections (Massa et al, 2009; Patel et al, 2007). Respiratory syncytial virus (RSV) and Rhinoviruses are viruses often associated with AOM development (Henderson et al, 1982). *Streptococcus pneumoniae* (15- 44% of total AOM cases), *Haemophilus influenzae* (11-15% cases) and *Moraxella catarrhalis* (8% cases) are the common bacterial pathogens recovered from OM fluid culture and nasopharangeal fluids of AOM patients (Brook & Gober, 2000). Recurrent AOM (RAOM) episodes can occur in some children and is termed RAOM, which is defined as three or more episodes of AOM in six months or four or more episodes in 12 months. More than 80% of children have at least a single AOM episode before the age of three (Cripps et al, 2005; Teele et al, 1989a). Six or more

AOM episodes occur in 40% of children by the time they are seven years old (Casselbrant et al, 1999). Unsurprisingly, AOM is one of most common childhood illnesses and a major reason for antibiotic prescription. A study by Paradise et al., shows that AOM is the reason for which children spend a mean of 42 d on antibiotics in the first year of life (Paradise et al, 2005).

1.1.1.2- Otitis Media with Effusion (OME)

OM can also manifest as OME in patients. OME usually follows an AOM episode and presents itself as middle ear inflammation without the symptoms commonly associated with AOM, i.e. ear pain and fever. The middle ear gets filled with effusion that is serous in nature and with a low cellularity of neutrophil leucocytes (NLs). However, over time the effusion can become non-serous or thick and glue-like (hence the name "glue ear"). OME is distinct from AOM in that its main symptoms of AOM (ear pain and fever) are absent in OME. Otoscopy studies have revealed that the tympanic membrane in OME can become retracted (Bluestone, 2004). OME can become chronic in some children, which is termed as chronic OME (COME), and may or may not lead to the development of chronic suppurative OM. OME and COME affected children develop conductive hearing loss, which may affect speech development (Bluestone & Klein, 2007).

1.1.1.3- Chronic Suppurative OM (CSOM)

CSOM is one of the most common childhood chronic diseases affecting a range of demographic sections across the world. CSOM is defined as the "chronic inflammation of the middle ear and mastoid mucosa with perforation in the tympanic membrane and presence of otorrhea" (Verhoeff et al, 2006). World Health Organisation (WHO) defines CSOM as "otorrhea

through a perforated tympanic membrane present for at least two weeks". Some authors classify as 'chronic' cases in which symptoms persist for at least 3-6 weeks (Bluestone 1998; Roland 2002). CSOM is different from COME in that perforation of the tympanic membrane is observed in CSOM along with highly suppurative exudate with a high number of NLs (Bluestone, 1998). Cholesteatoma or accumulation of kertanised stratified squamous epithelium in the middle ear can sometimes occur in CSOM. Such cases are termed CSOM with cholesteatoma.

CSOM can result in conductive hearing loss between a range of 20 to 60 dB (Kaplan et al., 1996). Studies have also shown that CSOM can cause sensorineural hearing loss due to the loss of hair cells in the cochlea (Cureoglu et al, 2004; Huang & Lin, 1991). CSOM-related hearing impairment can adversely affect children's language and psycho-social development (Winskel, 2006).

CSOM mostly occurs in the first five years of life in children (Wintermeyer and Nahata, 1994). A high prevalence of CSOM has been observed in children with craniofacial abnormalities (Bluestone, 1998) and certain racial groups such as Aborigines, Inuits and American Indians (Coates, 2002; Couzos et al, 2003). Despite the low prevalence of CSOM in the UK and U.S. (<1%), it is a significant burden on the healthcare system. The WHO has reported that a prevalence of CSOM >4% in a defined population indicates a massive public health problem in need of urgent attention (WHO, 1998) (Acuin, 2004). Roughly 51,000 deaths occur worldwide each year due to CSOM complications such as lateral sinus thrombosis, cerebral abscess, otitic meningitis etc. The WHO also estimates that 65 million to 330 million individuals develop CSOM, with subsequent hearing impairment in 60% of affected individuals (Acuin, 2004).

1.1.2- OM medical management

OM is medically managed with antibiotic and steroidal therapies, which are not very effective in OME (Browning et al, 2010; Griffin et al, 2006). While the majority of children are able to resolve OM, others suffer from persistent inflammation in the form of chronic OM. No effective medical management therapies for COME or CSOM currently exist. Chronic OM is treated with surgical intervention the most common of which is via the insertion of a tympanostomy tube otherwise known as ventilation tube or grommets, to ventilate the middle ear (Antonelli, 2004). Approximately 30,000 ventilation tube insertions are carried out in the UK each year as a surgical intervention treatment for chronic OM (Sood & Waddell, 2007). This surgical intervention offers only short-term hearing improvement in OME-effected children and can result in secondary complications such as otorrhoea, ear infection and loss of hearing in the operated ear (Browning et al.,2010, Antonelli, 2004).

Multiple patho-genetic factors might be responsible for the increased persistence of middle ear inflammation in some children and the resolution of OM in others. OM has a strong genetic component (see below), but the underlying genes involved in the development of persistent OM need to be explored.

OM risk factors

OM is a multi-faceted disease with a number of factors contributing to its development. Various environmental and host genetic factors have been linked to susceptibility to OM.

1.2.1- Socio-environmental factors

Socio-environmental conditions such as poverty, overcrowding, malnutrition and exposure to cigarette smoke have all been associated with the perpetuation of OM ((Elemraid et al, 2009; Lasisi et al, 2007; Uhari et al, 1996). Daycare attendance by children (Waldeb et al.,1989) has been shown to increase drastically the susceptibility of children to OM. Short-term breastfeeding , exposure to pollutants and premature birth have all been reported to increase the risk of developing OM (Brauer et al, 2006; Engel et al, 1999a; Engel et al, 1999b; Xenellis et al, 2005). The risk of developing RAOM and chronic OM increases by up to 66% in the presence of passive parental cigarette smoke ((Damoiseaux et al, 2006; Uhari et al, 1996).

1.2.2- Pathogenic Factors

Risk factors for the development of chronic OM include infection with AOM pathogens (Vergison, 2008). AOM is a multi-pathogen disease caused by bacteria and viruses. A triad of commensal bacteria – *S. pneumoniae,* non-typeable *H. influenzae* (NT*Hi*) and *M. catarrhalis* – are associated with 80% of AOM cases (Vergison, 2008). 20% of AOM cases are attributed to viruses alone (Segal et al, 2005). Coronavirus, respiratory syncytial virus (RSV) and adenovirus are the viruses most commonly associated with AOM (Chonmaitree et al, 2008).

1.2.3- Biofilms

Biofilms are complex communities of bacteria in matrix-enclosed biopolymer bacterial aggregates; these are often associated with chronic diseases such as endocarditis, cystic fibrosis, periodontal disease (Donlan & Costerton, 2002; Homoe et al, 2009). The common bacteria found in CSOM – *Pseudomonas aerugionosa, H. influenzae, Pneumococcus* spp. etc. – are known to form biofilms. Biofilms have been found in the middle ear of chinchillas with COME (Homoe et al, 2009; Jurcisek et al, 2005; Swords et al, 2004). The presence of *H. influenzae, S. pneumoniae,* and *M. catarrhalis* biofilms has also been demonstrated in mucosal lining from children with COME or RAOM (Hall-Stoodley et al, 2006). In a study on a high-risk CSOM population in Greenland, morphological evidence of biofilms in otorrhea in five of six children with CSOM has been observed (Homoe et al, 2009). Middle ear biopsies from adults operated for CSOM have also demonstrated the presence of biofilms (Homoe et al, 2009). The role of biofilms in OM pathogenesis is relatively unknown; whether biofilms are the cause or sequelae in middle ear diseases is yet to be established.

1.2.4- Host factors

1.2.4.1- Anatomical defects -Craniofacial disorders and Eustachian tube

Risk factors for OM also include anatomical defects such as craniofacial defects and Eustachian tube (ET) abnormalities. CSOM is over-represented in children with Down's syndrome, cleft palate and craniofacial disorders. Paradise et al. (1969) first demonstrated the near universality of OM in cleft palate patients. 98% of cleft palate patients develop OME and hearing loss by the age of five, according to a recent study performed on 86 cleft palate patients (Szabo et al, 2010). Cleft palate is a developmental abnormality;

TGF-β signalling is known to be involved in developmental processes including development of palate (Proetzel et al, 1995).

Many authors have also ascribed CSOM development to ET dysfunction (Bluestone et al, 2005; Sade & Fuchs, 1997). The ET links the naso-pharynx to the middle ear and is involved in pressure regulation, ventilation and the removal of secretions and dead cells (Bluestone, 1996). Children with craniofacial abnormalities such as cleft palate have a shorter ET and are more prone to ET obstruction and OM development. However, it is argued that ET obstruction is not the primary cause of OM but is instead a secondary event due to formation of mucus after inflammation has initiated (Sade & Fuchs, 1997). However, there is insufficient evidence in support of ET as the main aetiological cause of OM in both syndromic and non-syndromic children, with the claim being based on incomplete evidence (Bhutta, 2011; Sade & Ar, 1997).

1.2.4.2- Genetic factors

A number of epidemiological reports over the last few years reveal a substantial OM burden worldwide. The Centre for disease control (CDC) estimates that OM accounts for over 20 million visits to physicians per annum in the U.S. (CDC, 2007). The burden of OM is significantly high, with evidence of chronicity in certain populations and races. The prevalence of CSOM was found to be 12%, 2.5% and 15% respectively in community-based epidemiology studies performed in Bangladesh (Biswas et al, 2005), Nigeria (Amusa et al, 2005) and Australia (Morris et al, 2005). The rate of OM incidence is unusually high in American Indians, Australian Aborigines (84%) and Inuits (81%), according to reported studies (Ayukawa et al, 2004; Coates, 2002; Gunasekera et al, 2007). High CSOM rates have also been

reported in Australian Aborigines and Inuits (25-45%), but with low incidence of choleosteatoma (1-3%) (Jassar et al, 2006). Other studies have also reported high hearing screen fail rates among 19% of 5-6 year-old Inuits (Ayukawa et al, 2004). One third of aboriginal children develop unilateral or bilateral hearing loss (Thorne et al, 2004). These differences in incidence, severity and complication rates of OM and CSOM between populations suggest a genetic contribution to OM development. Studies of OM in Apache Indians in Arizona have also suggested familial disposition (Todd, 1987). Various studies suggest that both RAOM and CSOM are 60-70% heritable (Casselbrant et al, 1999; Casselbrant et al, 2009; Kvaerner et al, 1996; Kvestad et al, 2004; Rovers & Zielhuis, 2004). Twin and triplet studies have reported that genetic traits play a major role in OM development and that OM susceptibility is inherited (Kvaerner et al, 1999). Substantial heritability of OM has been replicated in a study from Norway including more than 9000 twins (Kvestad et al, 2004). A number of inflammatory gene polymorphism association studies have identified polymorphisms in innate immune system related genes such as *IL-6, TNF-α, IL-10, TLR-4, IL-1β* are associated with OM development (Daly et al, 2004; Emonts et al, 2007a; Patel et al, 2006; Pettigrew et al, 2006; Sale et al, 2011). However, our knowledge of the genetic susceptibility factors involved in the chronic progression of OM is still limited. There is a need to explore and further identify the genes that might be involved in OM.

OM Models

Studies outlined above suggest a strong genetic component in OM development. Mice can play a key role in undertaking investigations of the genetic and cellular pathways that may be involved in the development of OM. Mice have been used as a model organism for a number of other diseases due to the physiological comparability with humans. Mouse models can be generated relatively quickly and are easily manipulated which is an added advantage. There is also over 90% synteny between the mouse (Mouse Genome Sequencing Consortium *2002*) and human (International Human Genome Sequencing Consortium *et al. 2001*; Venter *et al. 2001*) genomes, which makes mice an excellent choice for modelling diseases.

Several murine models of OM have been described in the literature. These models allow a greater understanding of the relationship between genes and disease susceptibility (Parkinson & Brown, 2002) and allow the identification of candidate genes. Such candidate genes may also be identified in with genome-wide association studies in humans. Mouse models also function as a suitable platform for drug discovery and development of non-surgical interventions and therapies for the treatment of OM in humans.

These OM mouse models can be either pathogen-induced models, allowing us to study the inflammatory mechanisms involved in OM that are due to host abnormality, which allows for further understanding the pathways involved in disease progression.

1.3.1- Pathogen challenge-induced OM mouse models

Inflammation is mediated by the innate immune system following activation of toll-like receptors (TLRs) by bacterial pathogen-associated molecular patterns (PAMPs) or immune complexes (Aderem & Ulevitch, 2000; Akira, 2001; Sabroe et al, 2003). TLRs are expressed primarily on macrophages, NLs and vascular endothelial cells of the middle ear (Pugin et al, 1993; Shuto et al, 2001). Binding of PAMPs to TLR elicits the activation of transcription factors such as nuclear factor-kappa beta (NF-κB), which activates a number of pro-inflammatory genes (Medzhitov & Janeway, 1998; Tak et al, 2001). Many of these genes encode for inflammatory cytokines (Ghaheri et al, 2007). Some of these cytokines, such as TNF-α, IL-1β and IL-8, have been identified in 77-91%, 67-97% and 92-100% of chronic middle ear effusions respectively (Kubba et al., 2000). Hence, the link between OM and these inflammatory cytokines is studied extensively by means of injecting bacteria or bacterial products in middle ear (MacArthur & Trune, 2006; Ryan et al, 2006).

Several induced models of OM have been reported, including chinchilla (Giebink, 1999; Gitiban et al, 2005) and rat (Clark et al., 2000). However, mice are the animals of choice to study OM, as they have been used in the past studies to study inflammation mediators and immune response to key OM-causing pathogens, such as non-typeable *Haemophilus influenzae* (NT*Hi*), *H. influenzae* type b, *P. aeruginosa*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* (Cripps et al, 1994; Green et al, 1993; Loeb, 1987; Yamamoto et al, 1997). The mouse is the premiere mammalian model system used in biomedical research. The most significant advantage in using murine OM models over other organisms is the availability of genetically

inbred mice in comparison to other outbred species along with the ease of genetic manipulation and availability of the mouse genome (Parkinson & Brown, 2002). Another advantage of using mice is the existence of strains with natural or induced mutations that exhibit spontaneous OM due to compromised defence of the middle ear. Also, unlike other animal models that display a lack of middle ear infection by *M. catarrhalis,* OM is observed in mice after *M. catarrhalis* inoculation (Melhus & Ryan, 2003).

The mouse middle ear, just as in humans, changes drastically in response to infection and inflammation. These changes include the generation of effusion, inflammatory cell infiltration and mucosal cell proliferation (Ebmeyer et al, 2005). The limitation of using mice as an OM model is that the mouse middle ear small in comparison to other animal models. In fact, the mouse middle ear has only a volume of ~ 5-6 µl (Huangfu and Saunders, 1983) which makes OM assessment difficult. However, this handicap is progressively being overcome by more sensitive OM assessment techniques, such as oto-endoscopy (Bhutta et al, 2010).

There are various methods of OM induction by pathogens. One is to challenge the nasal mucosa with OM pathogen or inflammatory substance. Once the substance/pathogen transits to the ET, it can result in OM (Sabirov et al, 2001). This method mimics the natural mode of middle ear (ME) infection, but the OM incidence in this case is sporadic and the dose of inducing agent to reach the middle ear is uncontrolled (Ryan et al, 2006). Alternatively, a controlled dosage is achieved via direct injection into the middle ear via the tympanic membrane (Krekorian et al, 1990). However, the resulting hole in the tympanic membrane poses a risk of contamination and fluid drainage. This risk can be circumvented by injection through the bulla which has reduced contamination and fluid drainage, however; as the

bulla is next to major vessel extreme care needs to be taken through the procedure.

Mice do not normally become infected with human OM bacteria, but human OM bacteria are extensively used to study OM in mouse models. It is interesting to note that susceptibility to the same bacteria can vary in different mice strains (Melhus & Ryan, 2003). For example, BALB/c is more susceptible to induced to OM than C57BL/6 mice (Melhus & Ryan, 2003). Non-infectious induced models of OM for understanding OM pathogenesis are also quite widely used. OM in such models is induced by injection of heat-killed bacteria, LPS or other inflammatory mediators such as TNF-α or IL-1β (Catanzaro et al, 1991; Krekorian et al, 1990; Maeda et al, 2004). These models may not model infection as closely as pathogen-based induced OM models, but they are useful for studying individual components of OM.

Although induced models of OM are useful and essential for understanding the pathogenesis of OM, their use is limited in addressing the genetic component of OM as a disease and the basis for differences in OM susceptibility that is observed in different demographic groups in humans. However, they are often used in conjunction with genetic models to induce chronic OM (Described in section 1.3.2).

1.3.2- Gene-driven mouse models of OM

Independent lines of evidence have indicated the role that host genetics might play in OM. Racial differences in the incidence of OM and CSOM suggest a genetic contribution of OM (Casselbrant et al, 1999; Coates et al, 2002; Emonts et al, 2007b; Todd, 1987). OM susceptibility is exhibited in various congenital and inherited syndromes, such as Achondroplasia, as

well as in patients with Down's syndrome, cleft palate or inflammatory gene polymorphisms (Casselbrant et al, 1999; Daly et al, 2005; Emonts et al, 2007a; Patel et al, 2006).

Studying the genetic pathways involved in human diseases is fraught with practical difficulties, such as uncontrollable environmental factors and a mixed genetic background. In such a scenario, mice can play a key role in understanding OM pathogenesis. The functional and anatomical similarity between the ears of mice and humans (except for the absence of mastoid air cells in rodents), their comparable physiology, and the availability of both murine and human genomes ((Lander et al, 2001; Venter et al, 2001; Waterston et al, 2002) make mice an excellent model for studying the genetics of OM (Brown et al, 2008). The murine genome is easily manipulated and mice can be generated to produce litters in 9 wks (Rye et al, 2010). The availability of tools to create knockout and transgenic mice along with easily inducible large-scale mutagenesis and phenotypic assessment of mutant strains has made mice a perfect model organism to study OM (MacArthur & Trune, 2006).

In gene-driven OM mouse models, gene-driven approaches such as gene traps, knockouts or knock-in mutations are introduced in mouse genomes to target specific candidate genes (Brown et al, 2008). This approach can be scaled up to target every gene in the genome. Programmes for the generation of null and conditional null lines is also underway and will further enable a comprehensive understanding of genes involved in OM (Auwerx et al, 2004). EUCOMM (European Conditional Mouse Mutagenesis program), which began in 2006, and IKMC (International Knockout Mouse Consortium) are similar programs to develop conditional

targeted knockouts for every gene in the mouse genome (Collins et al, 2007).

1.3.2.1- Deletion of *E2f4* gene results in OM in mice

E2F transcription factors are key regulators of the cell cycle and control the expression of genes required in the G1/S transition (Helin, 1998). Mutation in the E2F gene has been previously associated with adult T cell leukemia in humans although no association with OM in humans has yet been established (Komatsu et al, 2000). Homologous recombination has been used to knock out the E2f4 gene and its DNA binding domains in *E2f4*$^{-/-}$ mice (Humbert et al, 2000). Although most mice die shortly after birth, investigations have revealed indications of OM and chronic rhinitis in these mutants along with other abnormalities such as craniofacial defects and erythroid cell abnormalities. (Humbert et al, 2000). *E2f4*$^{-/-}$ mutants are found to be infected with *Pasteurella pneumotropica* along with the accumulation of purulent fluid due to macrophage and NL infiltration at the site of inflammation. *E2f4*$^{-/-}$ also have mild craniofacial abnormalities along with atrophic olfactory epithelium and mucus accumulation in the nasal passage and a lack of ciliated cells in the postnatal epithelium (Danielian et al, 2007). However, ME exudate was still found to be present in the bulla of *E2f4*$^{-/-}$ homozygous mice even under *Pasteurella pneumotropica* free conditions. Inflammatory responses were not found to be affected in *E2f4* deficient mice, which suggest structural changes as the cause behind the increased bacterial susceptibility observed in these mutants. The evidence outlined above suggests that OM in *E2f4*$^{-/-}$ mutants is due to a defect in embryonic development of cilia resulting in the production of mucin, which

accumulates in the middle ear and nasal passage to cause OM and chronic rhinitis respectively (Rye et al, 2011a).

1.3.2.2- Mice deficient in transformation-related protein p73 gene display AOM symptoms

In this mutant the *transformation-related protein 73* (*p73)* gene, was disrupted in embryonic cells (Yang et al, 2000). The *P73* gene encodes a member of the p53 family of transcription factors and is involved in cellular responses to stress, cell differentiation and inflammation (Moll & Slade, 2004). *P73* shares sequence homology with the gene for transcription factor and tumour suppressor *P53,* but is not involved in oncogenesis (Fontemaggi et al, 2002).

Chronic inflammation and infection have been observed in mice deficient in *p73* along with other larger defects such as hippocampal dysgenesis, congenital hydrocephalus and other growth defects as well as high mortality rate (Yang et al, 2000). $p73^{-/-}$ mice develop purulent OM 2 d post-birth (DAB), which continues through adulthood with ≥80% of adult mutants displaying AOM symptoms along with NL accumulation at the site of inflammation. Bacteria such as *E. coli* and *Pasteurella aerogenes* were isolated from the middle ear at 21 DAB but not 2 DAB. Chronic inflammation is observed in $p73^{-/-}$ mice while no lymphoid or granulocyte deficiencies or any craniofacial abnormalities were found (Yang et al, 2000). This hints at a defect in other immune system components in $p73^{-/-}$ mice, such as hyper-production of mucus and inflammation of the mucous membrane, which might make these mutants susceptible to bacterial infection (Rye et al, 2011a). The human orthologue of *p73*, *TP73* has also

been found to be associated with chronic rhinosinusitis (CRS) and OM in humans (Bosse et al, 2009; Tournas et al, 2010).

1.3.2.3- *Lpr/lpr* mice expressing defective Fas protein display prolonged AOM

Lpr/lpr is a homozygous recessive lymphoproliferation mutation model that exhibits Lupus-like autoimmune disorder due to a defective apoptosis receptor Fas protein (Watanabe-Fukunaga et al, 1992). Mutations in *FAS* gene are associated with diseases such as autoimmune lymphoproliferative syndrome and pulmonary fibrosis in humans (Nagata, 1997; Tafani et al, 2002). An insertion of a transposable element in the *Fas* intron results in defective Fas protein (Watanabe-Fukunaga et al, 1992).

Fas is a member of the TNF superfamily and is involved in apoptotic signalling (Siegel et al, 2000). The *lpr/lpr* mutation results in a systemic autoimmune mutation on the MRL background and OM induction by *Salmonella typhi* LPS results in a continued mucosal hyperplasia and inflammatory cell infiltration in the middle ear even after 14 d, while the hyperplasia is observed in control mice only up to 5 d (Rivkin et al, 2005). This significant difference in the recovery of OM-affected middle ear mucosa suggests a role of Fas in prolonging OM and the repair of mucosa.

1.3.2.4- Toll-like receptor defects and OM

Toll-like receptors (TLR) are key innate immune system regulators and crucial for elimination of bacterial pathogens. They are a type of pathogen recognition receptors (PRRs) present on immune cells such as macrophages, dendritic cells (DC), monocytes and B lymphocytes which recognise PAMPs on bacteria to trigger the signalling pathways involved in the

production of cytokines essential for pathogen clearance (Takeda & Akira, 2003; Takeda et al, 2003). TLR defects have been shown to be associated with OM in mouse models (Rye et al, 2011a).

A single amino acid change causing the mutation in the *Tlr4* gene renders C3H/HeJ mice susceptible to spontaneous chronic OM, along with conductive hearing loss, at around 5-12 months of age (MacArthur et al, 2006). C3H/HeJ mice have normal craniofacial structure and ET, which suggests that the OM observed in these mice is due to the loss of function of *Tlr4*, which results in a defective bacterial clearance in these mutants (MacArthur et al, 2006). Despite being a spontaneous OM model, C3H/HeJ mice do not develop OM until around 5 months of age, which suggests that this model may not be biologically relevant as a model of OM in childhood (Rye et al, 2011a). However, injecting NT*Hi* into the tympanic cavity in C3H/HeJ and WT control C3H/HeN results in AOM in both genotypes (Hirano et al, 2007). However, though WT mice resolve inflammation within 24 h, OM in C3H/HeJ persisted past 72 h. This result, along with the immune-histochemistry staining of the tympanic membrane, indicated an important role of Tlr4 in the resolution of OM caused by bacterial infection (Hirano et al, 2007).

Interestingly, TLR4 polymorphism in humans has also been associated with RAOM and COME/RAOM in studies (Emonts et al, 2007a; Sale et al, 2008).

The targeted disruption of the *Tlr2* gene also shows a delayed bacterial clearance (Leichtle et al, 2009). AOM inducted by NT*Hi* injection into the tympanic membrane of $Tlr2^{-/-}$ mice persisted at least 10 d in comparison to WT mice, which resolved the AOM within 2 d. No association between

TLR2 and RAOM/COME has been observed in human studies (Sale et al, 2008).

1.3.2.5- Myeloid differentiation primary response protein 88 defect display chronic AOM on bacterial challenge

The myeloid differentiation primary response protein 88 (MyD88) is an adaptor molecule involved in TLR signalling. Targeted disruption of *MyD88* was performed to generate *MyD88*$^{-/-}$ knockout mice on C57BL/6J background (Hernandez et al, 2008). Induction of AOM by NTHi injection into the tympanic membrane of *MyD88*$^{-/-}$ mice resulted in chronic AOM, which persisted for at least 21d in comparison to WT mice, which resolved the inflammation in 5 d.

Delayed inflammatory cell recruitment was observed in *MyD88*$^{-/-}$ mice. Defective and delayed inflammatory cell recruitment predisposes MyD88-deficient mice to inflammation due to the defective clearance of bacteria (Rye et al, 2011a). In humans, the autosomal recessive mutation in *MyD88* predisposes the affected individuals to certain bacterial populations, especially *S. pneumoniae,* which is a key OM-causing bacterium (von Bernuth et al, 2008).

1.3.2.6- Plasminogen deficient mice display fibrin deposition and OM

Spontaneous chronic OM has also been reported in *plasminogen* knockout mice (Eriksson et al, 2006). Plasminogen is a serine protease involved in wound healing, fibrinolysis and inflammatory cell migration (Bugge et al, 1995; Ploplis & Castellino, 2000). It is encoded by the *Plg* gene. *Plg*-deficient mice displayed fibrin deposition in a number of mucosal surfaces and organs, including the tympanic cavity, along with other inflammatory

changes such as infiltration of neutrophils and macrophages, and abnormal keratin formation (Eriksson et al, 2006). Variable numbers of bacteria were also isolated from the tympanic cavity of these mice. Polymorphisms in the plasminogen activator inhibitor 1 gene *PAI-1* polymorphisms have been associated with RAOM in human GWAS studies, which suggests a role of the plasminogen pathway in OM pathogenesis (Emonts et al, 2007b).

1.3.2.7- Eyes absent homolog 4 gene deficient mice develop spontaneous OME

Eya4 knockout mice $^{-/-}$ develop spontaneous OME regardless of genetic background (CBA/J, BALB/c, C57BL/6 or Swiss-Webster) (Depreux et al, 2008). The *Eya*$^{-/-}$ mice develop OME by 16 DAB and the retraction of the tympanic membrane is evident by 21 DAB. Craniofacial and anatomical analyses have suggested that OME development in these mice may be due to an abnormal middle ear cavity and ET dysmorphology. However, *Eya4* has also been found to play an important role in apoptosis and innate immune system regulation as a transcription factor , providing an alternative explanation to the chronic and spontaneous OM observed in these mice (Okabe et al, 2009).

1.3.3- Phenotype-driven mouse models of OM

In the phenotype driven approach, randomly mutagenized mice are screened for disease phenotypes (Nolan et al. 2000). Usually this mutagenesis is performed using is by ENU (N-ethyl-N-nitrosourea) which is a potent mutagen that induces random point mutations in spermatogonial stem cells (Justice et al, 1999). This method follows a forward genetics approach in which a phenotypic screens are performed to identify disease phenotypes of

interest (Hrabe de Anglelis et al.,2000; Nolan et al.,2000). This way the abnormal phenotype of interest is starting point of the investigation. Genetic mapping allows positional cloning of the causative mutation.. This method does not make prior assumptions about the relationship between the phenotype and gene and is useful tool to discover novel genes involved in complex diseases such as OM. ENU induces point mutations in individual loci to give null, gain or loss of function mutations. Strains bearing separate mutations can be crossed to make double mutants and understand multi-factorial basis of disease (Schachern et al, 2007; Zheng & Johnson, 2001).

Phenotypic assessment of these mutants is performed using procedures such as click box, which measures preyer reflex to loud noise (90dB) in mice identifing mutants with conductive hearing loss or sensorineural hearing defects ((Hardisty et al, 2003; Nolan et al, 2000). Another more sensitive method to measure hearing thresholds in mice is through auditory brainstem response (ABR) (Zheng et al.,1999; Hardisty-Hughes et al.,2010). In ABR, defined sound frequencies are played into the ear of the mouse and the brainstem response to the stimulus is then monitored to determine the hearing threshold in decibels (dB). The stimulus range is 90 dB to 5 dB. Another diagnostic method for OM assessment is by a medical imaging technique for middle ear examination or oto-endoscopy which has a 93% accuracy in OM diagnosis in mice (Bhutta et al.,2010). In combination with ABR and click-box, mouse oto-endoscopy presents an efficient and reliable method of OM assessment in mouse mutants.

Phenotypic screening of ENU generated mutants led to discovery of 2 novel mouse mutants *Jeff* and *Junbo* at MRC Harwell (Nolan et al, 2000). Both *Jeff* and *Junbo* display a conductive deafness phenotype characterised by middle ear fluid and inflammation and lack of preyer reflex in click box test

(Hardisty-Hughes et al, 2006; Parkinson et al, 2006). These mouse mutants are the first good spontaneous mouse models for chronic OM that mimic the phenotype of the human condition.

Table 2.1- Current models of OM in mice

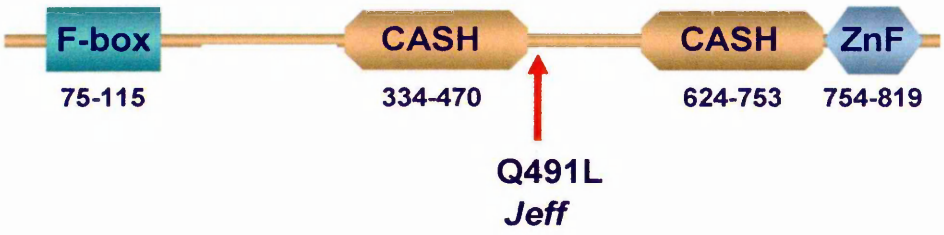
Gene	Chromosome	Model type	OM Phenotype	Reference
E2f4	10	KO	Spontaneous chronic	Humbert *et al.* 2000
Evi-1	3	SNP	Spontaneous chronic	Parkinson *et al.* 2006
Eya4	10	KO	Spontaneous chronic	Depreux *et al.* 2008
Fbxo11	17	SNP	Spontaneous chronic	Hardisty-Hughes *et al.* 2006
P73	4	KO	Spontaneous chronic	Yang *et al.* 2000
Plg	17	KO	Spontaneous chronic, fibrin deposition	Eriksson *et al.* 2006
Fas	19	SNP	Induced acute	Rivkin *et al.* 2005
MyD88	9	KO	Induced acute	Hernandez *et al.* 2008
Tlr2	3	KO	Induced acute	Leichtle *et al.* 2009
Tlr4	4	SNP	Induced acute	Hirano *et al.* 2007

This table describes the various current OM models discussed in this thesis along with the affected genes. Adapted from (Rye et al, 2010) KO knockout, SNP Single nucleotide polymorphism.

1.3.3.1- Non-syndromic spontaneous chronic OM model- *Fbxo11* mutation in *Jeff*

Jeff is a dominant mouse mutant displaying OM phenotype similar to COME in humans. The *Jeff* mice carry a point mutation in F box gene *Fbxo11* (Figure 1.2) (Hardisty-Hughes et al, 2006). *Jf/+* mice display a conductive hearing loss along with a middle ear cavity filled serous fluid effusion by 28 dab (Hardisty-Hughes et al, 2006; Hardisty et al, 2003). Cellular hypoxia in inflammatory cells in middle in *Jf/+* middle ear has been observed (Figure 1.3) (Cheeseman et al, 2011). *Jf/+* mice do not have any obvious immune deficiencies but display an OM and deafness phenotype along with mild craniofacial abnormalities, shortened snout and narrow bent ET (Hardisty et al, 2003). However, *Jf/+* mice on a C57BL/6

unlike the original C3H/HeH background do not develop craniofacial defect but still develop OM.



H.sapiens	NALAGIQIRTNSCP
M. Musculus	NALAGIQIRTNSCP
Jeff Fbxo11	NALAGILIRTNSCP
R. norvegicus	NALAGIQIRTNSCP
G. Gallus	NALAGIQIRTNSCP
D. rerio	NALAGIQIRTNSCP
F. rubripes	NALAGIQIRTNSCP
D. Melanogaster	NALAGIQIRSQSDP
A. Gambiae	NALAGIQIRTTSDP
C. Elegans	NALAGIQIRTNSDP
C. intestinalis	NALAGIQIRTNSSP

Figure 2.2- Fbxo11 sequence is conserved. Mutation in Fbxo11 sequence underlying the Jeff mutant phenotype

Adapted from (Hardisty-Hughes et al, 2006)

The *Fbxo11* mutation in *Jeff* mutant was mapped to chromosome 17 (Hardisty et al, 2003). The mutation in *Jeff* is at a highly conserved portion of the gene at amino acid 491 of *Fbxo11* gene from glutamine to leucine (Hardisty-Hughes et al, 2006). *Fbxo11* is expressed in bone marrow, tissue macrophages, epithelial cells of middle ear from late embryonic stages as well as in liver and kidney after birth (Hardisty-Hughes et al, 2006). *Fbxo11* is part of a large family of F-box proteins which function as a part of SKP1-

cullin-F-box (SCF) E3 protein ligase complex. These proteins promote proteosomal ubiquitination and degradation by recognizing and binding to phosphorylated proteins (Jin et al, 2004; Kipreos & Pagano, 2000).

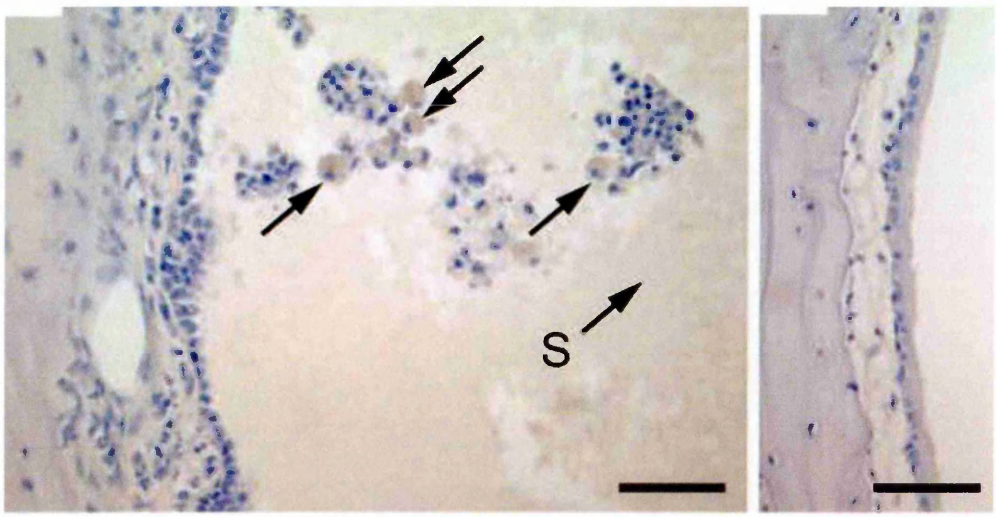


Figure 2.3- Inflammatory cells in *Jf/+* middle ear effusion are hypoxic.

Left panel shows PIMO labeling in *Jf/+* inflammatory cells in the bulla lumen (arrows) arrow S indicates serous fluid. Right panel shows WT mucosa where PIMO labeling is negative. Scale bare denotes 50 μm. Picture adapted from (Cheeseman et al, 2011).

Jf/Jf homozygotes have been shown to have palatal defects and eyes-open at birth (EOB) phenotype (Hardisty-Hughes et al, 2006; Tateossian et al, 2009). TGF-β has been known to be involved in all these processes (Pelton et al, 1991; Prime et al, 2004; Zhang et al, 2003). Phosphorylated Smad2 (pSmad2) is an intracellular signalling protein in the TGF-β pathway and is upregulated in epithelia of *Jf/Jf* homozygotes in the eyelids, palates and lungs. Interaction between Fbxo11 and Smad2 has also been assessed using compound heterozygous *Jf/+Smad2/+* mutants which had a phenotype similar to *Jeff* homozygotes. However no biochemical interaction between the two proteins has been observed (Tateossian et al, 2009). This suggests that Fbxo11 might be indirectly interacting with Smad2 protein and be

involved in TGF-β signalling regulation. Recent literature has revealed that Fbxo11 protein acts as a Nedd8-ligase to p53 (Abida et al, 2007). siRNA knockdown and overexpression experiments have shown that neddylation of p53 by Fbxo11 reduces the transcriptional activity of p53 but does not affect its stability (Abida et al, 2007). However, a co-immunoprecipitation study performed on *Jeff* mice failed to reveal any direct interaction between the two proteins (Tateossian et al, 2009). Although *Jf/Jf* homozygotes and *Jf/+* heterozygotes had markedly reduced and intermediate p53 protein levels respectively in comparison to WT. This suggests that Fbxo11 maybe requisite for stabilization of p53 protein (Tateossian et al, 2009). p53 protein functions as a transcription activator and is maintained at low level in unstressed cells. In response to hypoxia, oxidative damage or oncogene activation, p53 levels and transcriptional activity is enhanced (Vogelstein et al, 2000). p53 is also known to directly interact with Smad2 to synergistically activate TGF-β target genes (Cordenonsi et al, 2003). p53 deficient mammalian cells demonstrate an impaired TGF-β response (Cordenonsi et al.,2003). It has been proposed that in *Jf/Jf* loss of *Fbxo11* function may result in p53 destabilisation by an unknown mechanism. p53 levels are substantially reduced in *Jf/Jf* homozygotes.

It is plausible that *Fbxo11* dysregulation in mutant *Jeff* impacts upon TGF-β signalling via Smad2 and p53, identifying TGF-β/SMAD pathway in OM susceptibility. This might result in an abnormal innate immune response resulting in persistent COME observed in *Jf/+* mice. Interestingly, evidence of association between human orthologue FBXO11 and COME/RAOM has also been observed in studies with FBXO11 SNPs in human OM families confirming a role of FBXO11 in OM pathogenesis (Rye et al, 2011b; Segade et al, 2006).

1.3.3.2- Spontaneous non-syndromic chronic OM model - *Evi-1* mutation in *Junbo*

Junbo mutant was identified as a novel OM model in a dominant ENU mutagenesis screen with preliminary phenotyping using a click box test (Parkinson et al, 2006). Heterozygote *Jbo/+* mutants exhibit conductive deafness phenotype due to middle ear effusion similar to CSOM observed in humans. However, no perforation of the tympanic membrane is observed in these mutants. Gross antatomical dysmorphologies are absent in *Jbo/+* mice. *Jbo/+* mutant develop spontaneous OM that transforms into persistent OM by 29 DAB, suggesting a predominant genetic susceptibility of *Jbo/+* mice to OM. These mice develop OM even in germ free conditions but the onset is delayed (unpublished data, Michael Cheeseman). This suggests that microbial infection is not necessary for initiation of OM but may accelerate its progression. *Jbo/+* mice have no obvious craniofacial abnormalities, overt immune defects or organ pathologies (Parkinson et al, 2006). Chronic middle ear effusions from *Jbo/+* were found to contain variable numbers of viable and necrotic NLs and foamy macrophages, bacterial colonies are observed infrequently. Labelling with the hypoxia marker pimonoidazole showed that middle ear in *Jbo/+* is hypoxic, with the epithelium, stromal cells, inflammatory cells in bulla lining all being hypoxic (Figure 1.4) (Cheeseman et al., 2011).

The underlying molecular basis of the *Jbo/+* phenotype is a mutation in the Ecotropic viral integration site 1 (*Evi-1)* gene (Figure 1.5). The mutation causes an A2288T transversion in the *Evi-1* locus, resulting in a non-conservative Asn763Ile change in the second of the two zinc-finger domains of the protein (Parkinson et al, 2006). *Evi*-1 gene was first identified as a retroviral integration site in mouse myeloid tumour model (Morishita et al,

1988; Mucenski et al, 1988a; Mucenski et al, 1988b). This gene is mapped to human chromosome 3q2q and its overexpression in humans is linked to myeloid malignancies such as acute myeloid leukemia (AML), chronic myeloid leukemia (CML), myelodysplastic syndrome (MDS) (Alessandrino et al, 2001; Mitani et al, 1994; Nucifora & Rowley, 1994; Sunde et al, 2006; Vinatzer et al, 2003). Evi-1 expression has been observed in myeloid and epithelial cells of middle ears without any genotypic differences between WT and *Jbo*/+ (Parkinson et al, 2006).

Evi-1 is a complex locus on mouse chromosome 3 (Nucifora et al, 2006). It encodes for a 145 kD transcriptional regulatory protein, and can act as a transcriptional activator as well as a repressor (Matsugi et al, 1990; Morishita et al, 1990b). The *Evi-1* gene is implicated in leukemia and the inhibition of TGF-β signalling (Mitani, 2004). The Evi-1 protein consists of 2 zinc finger domains with 7 zinc finger motifs in N terminal of zinc finger domain 1(ZF1) and 3 zinc finger motifs in zinc finger domain 2 (ZF2), a central transcription repression domain between ZF1 and ZF2 along with C terminal acidic region (Bartholomew et al, 1997; Morishita et al, 1990b). Apart from the full length 145 kD Evi-1 transcript which is the major Evi-1 form, other alternatively spliced forms of Evi-1 are known to exist in mice and humans (Wieser, 2007). Evi-1b is a 727 protein which lacks 324 amino acid residues coding for Zinc finger motifs 6 and 7 of ZF1 as well as adjacent C-terminal residue. It encodes for 88kD protein (Hirai, 1999; Morishita et al, 1990a; Morishita et al, 1990b; Morishita et al, 1988). It is also known as the Evi-1 Δ324 isoform (Goyama & Kurokawa, 2010). This isoform of Evi-1 is expressed in low levels in humans and mice (Wieser, 2007). Another Evi-1 mRNA variant (Δ105) encodes protein lacking 105

amino acid residues in C terminal. It is abundant in murine cells but not in humans (Alzuherri et al, 2006; Wieser, 2007).

The Rp9 variant is abundant in both humans and mice and the protein derived lacks 9 amino acid residues from the repression domain (Alzuherri et al, 2006). Apart from alternative splicing of the *Evi-1* gene, presence of alternative transcriptional initiation sites results in a transcriptional variant termed as *MDS1/EVI1*. This variant has an additional 188 amino acid residues derived from *MDS1* gene at the N terminal of Evi-1 protein (Fears et al, 1996; Wieser, 2007). These amino acids encode a PR domain which has been implicated in tumour suppression in humans while PR absent forms are considered oncogenic (Goyama & Kurokawa, 2010)

Apart from being implicated in leukemia, a role of *Evi-1* in development has also been suggested owing to its restricted expression pattern in embryo and different organs in mice (Wieser, 2007). Deletion of *Evi-1* by the disruption of 6^{th} exon confirms the gene's role in development. Homozygous *Evi-1*$^{-/-}$ mice demonstrate perinatal lethality along with hypocellularity, defects in limb development and nervous system and circulatory system (Hoyt et al, 1997; Wieser, 2007). Heterozygote mice did not display OM and were phenotypically similar to WT. This may be due to Δ324 isoform not being affected in the KO genotype (Parkinson et al, 2006).

Apart from its role in leukemia and development, one of the main biochemical functions of Evi-*1* is as a transcriptional activator and regulator. Only few target genes (*Gata2, Map3k14, Fog2)* are currently known (Takahashi & Licht, 2002; Yatsula et al, 2005; Yuasa et al, 2005). Evi-1 exerts these biochemical functions via its DNA binding zinc finger domains. ZF1 recognizes a consensus sequence of GACAAGATAAGAT (Delwel et al, 1993; Goyama & Kurokawa, 2010) while ZF2 recognizes consensus

sequence of GAAGATGAG (Morishita et al, 1995). Evi-1 also interacts with transcriptional corepressors such as CtBP and epigenetic transcription regulators Histone deacetylases (HDACs) (Chakraborty et al, 2001; Chi et al, 2003; Vinatzer et al, 2001) as well as transcriptional co-activators CBP and P/CAF (Chakraborty et al, 2001).

ZF1 binds directly to the *Gata-2* promoter (Yuasa et al, 2005). ZF1 also binds to Smad3 and cJun N-terminal kinase (JNK) (Kurokawa et al, 1998a; Kurokawa et al, 1998b; Kurokawa et al, 2000). Evi-1 exerts its repressive effect on Smad3 via its CtBP binding consensus motif containing repression domain between the 2 zinc finger domains (Izutsu et al, 2001; Kurokawa et al, 1998b). This links *Evi-1* to the TGF-β signalling pathway through its interaction with Smad3, suggesting that the mutation may cause OM through its effects on TGF-β signalling.

The ZF2 domain of Evi-1 binds *cFos* and induces activation of transcription factor Activator protein-1 (AP-1) which is either a Fos/Jun heterodimer or Jun/Jun homodimer (Tanaka et al, 1994). Evi-1 also stimulates PI3K/AKT signalling pathway in intestinal epithelial cells (Liu et al, 2006). AP-1 functions as a regulator of several cellular processes including differentiation, apoptosis and inflammation.

The role of *Evi*-1 in inflammation is not clear yet but both TGF-β and AP-1 pathways are known to crosstalk with numerous pathways involved in inflammation and immune defence, and it remains to be determined how the *Evi-1*^{A2288T} mutation leads to CSOM observed in *Jbo/+* mutants. Interestingly, the Minnesota COME/RAOM Family Study cohort failed to show association of *EVI-1* with OM (Rye et al, 2011a; Sale et al, 2008). However, no study on association of *EVI-1* with alternative OM phenotypes

such as CSOM has been performed yet and such studies may reveal an association (Rye et al, 2011a).

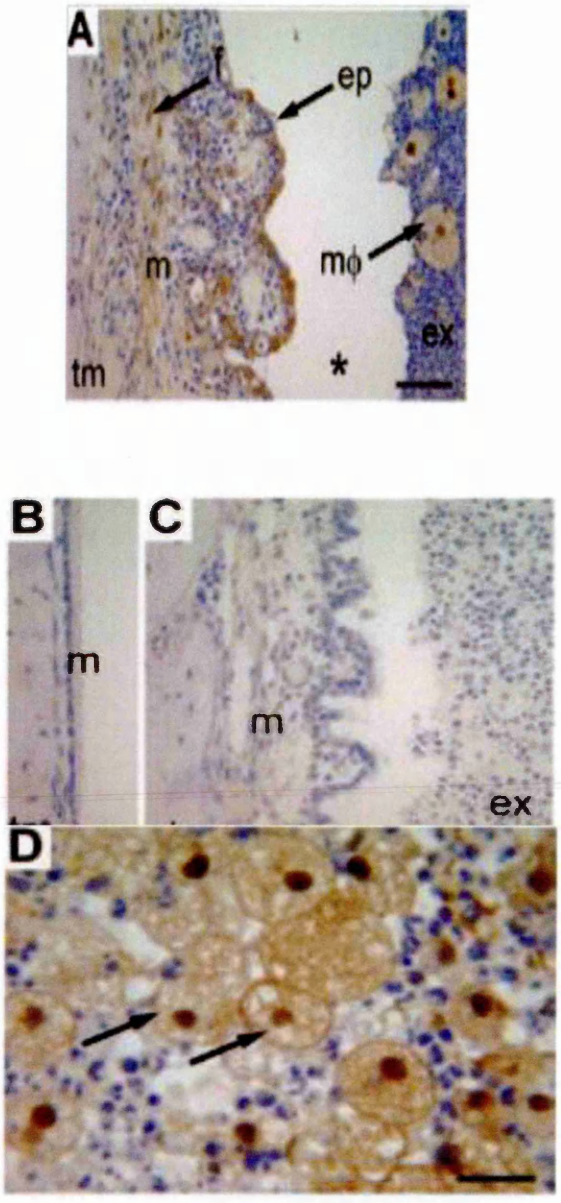


Figure 2.4- Inflamed middle ear in *Jbo/+* mice is hypoxic

Adapted from(Cheeseman et al, 2011). Panel A displays *Jbo/+* mice labeled with pimonidazole (PIMO), arrows indicating hypoxia in epithelium (ep), macrophages (mø), exudate (ex), temporomandibular bone (tm) and thickened mucosa (m). Panel B shows thin mucosa and absence of inflammation in *Junbo* WT mouse. Panel C is an unlabelled *Jbo/+* mouse. Panel D shows hypoxic foamy macrophages in *Jbo/+* ear exudates. Scale is 50µm.

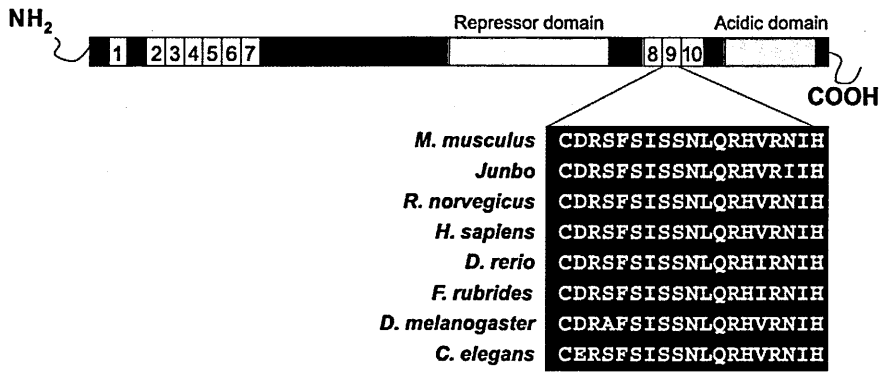


Figure 2.5- Evi-1 mutation in *Junbo* mice

Adapted from (Parkinson et al, 2006). Picture shows schematic representation of Evi-1 peptide with the 2 zinc finger domains separated by a repressor domain. Expanded peptide sequence shows the high degree of conservation at the region of mutated domain

Hypoxia and Inflammation

All mammalian cells have an intrinsic ability to sense and adapt to tissue oxygen levels and hypoxia. Hypoxia leads to activation of the hypoxia inducible factor (HIF) pathway which induces genes involved in restoring blood supply, nutrients, and energy production to maintain tissue homeostasis. HIF-1 is a transcription factor and global regulator of oxygen homeostasis (Semenza & Wang, 1992). Target hypoxia-responsive genes are involved in various cellular processes such as anaerobic respiration ascribed to the up-regulation of expression of glucose transporters (GLUT), angiogenesis by activating expression of pro-angiogenic factors such as VEGF, cell proliferation and apoptosis amongst others. HIF dependent hypoxia responsive gene expression also plays a key role in innate immunity and inflammation due to extensive cross talk between the HIF pathway and inflammatory pathways via NF-κB (Rius et al, 2008). Hypoxic responses in inflamed micro-environments is favourable for microbial clearance but can also exacerbate inflammatory damage depending on degree and duration of hypoxia. Pathologic hypoxia is found at inflammatory sites including

inflamed middle ear in *Jbo/+* and *Jf/+* where it creates a unique microenvironment condition affecting the function of cells and delivering important inflammatory signals (Cheeseman et al, 2011).

1.4.1- Regulation of HIF-1α and its activity

HIF-1α is the oxygen sensitive subunit of hetero-dimer HIF which consists of unstable HIF-1α subunit and ubiquitously expressed stable β aryl hydrocarbon translocator subunit HIF-1β (Jaakkola et al, 2001; Wang et al, 1995).

1.4.1.1- HIF-1α regulation by O_2 availability

HIF-1α is known to activate ≥ 100 hypoxia-responsive genes which are involved in various cellular processes (Semenza & Wang, 1992). Gene expression studies on WT vs *Hif-1α* knockout embryonic tissues have revealed 234 genes which are affected by loss of *Hif-1α* (Vengellur et al, 2003). Activation of these genes is dependent upon stabilization of oxygen sensitive HIF-1α subunit, making it a master regulator of hypoxic response. HIF-1α is a member of Per-ARNT-Sim domain family (PAS domain family) (Bracken et al, 2003; Weidemann & Johnson, 2008). It is a basic helix loop helix transcription factor. Regulation of HIF-1α subunit occurs at both protein and functional level through action of prolyl hydroxylase and prolyl asparaginyl enzymes (Lando et al, 2002; Masson et al, 2001). Under normoxic conditions, prolyl residues Pro402 and Pro 564 in the N-terminal domain of HIF-1α are hydroxylated by iron(II) and 2-oxoglutarate dependent prolyl hydroxylase enzymes (termed PHD1, PHD2, PHD3) (Epstein et al, 2001; Masson et al, 2001). This enables its binding to von Hippel-Lindau (VHL) to form VHL-E3-ubiqutin ligase complex, which then initiates HIF-1α ubiquitination and degradation by the proteosome (Ivan et

al, 2001; Jaakkola et al, 2001; Maxwell et al, 1999). During hypoxia, decreased hydroxylase activity leads to HIF-α subunit stabilization and nuclear translocation where it forms a complex with HIF-1β and its cofactors CREB binding protein (CBP) and p300. Inside the nucleus, the complex binds to the HIF-DNA consensus-binding sites or HREs of genes to drive an increased transcription of HIF-1α target genes (Lando et al, 2002; Masson et al, 2001).

In addition to the protein abundance, the transcriptional or functional activity of Hif-1α gene is also oxygen regulated (Jiang et al, 1997; Pugh et al, 1997). Under normoxia, Proline residue 564 of Hif-1α is hydroxylated by iron(II) and 2-oxoglutarate dependent dioxygenase, Factor Inhibiting HIF (FIH) binding to the proximal C-terminal transactivation domain (CAD) of HIF (Mahon et al, 2001) (Lando et al, 2002). This prevents the binding of HIF-1α to p300 which is a co-activator of HIF complex (Lando et al, 2002; Mahon et al, 2001), thereby affecting its downstream gene activation (Sang et al, 2002).

1.4.1.2- Hif-1α regulation via PHD regulation

Although oxygen is the main determinant of HIF-1α via PHD activity, the PHD enzyme is also sensitive to redox balances, iron homeostasis and ascorbate (Epstein et al, 2001; Ivan et al, 2002). HIF-1α is also known to negatively regulate its abundance by upregulating *PHD2* and *PHD3* gene transcription in hypoxia (D'Angelo et al, 2003b; Metzen et al, 2005). Increased PHD abundance may compensate for reduced hydroxylase activity and prepare cells to clear HIF-1α quickly once they are exposed to normoxic conditions again (D'Angelo et al, 2003a).

1.4.1.3- O_2 independent Hif-1α regulation

Hif-1α expression can be up-regulated by many growth factor and cytokines such as TNF-α, IL-1β. Nitric oxide (NO) produced by activated macrophages is one such mediator (Thomas et al, 2008). Exogenous addition of NO in normoxia is known to stabilize HIF-1α protein and transactivate HIF-1 under normoxic conditions. It does that by co-ordinating ferrous iron and decreasing PHD activity (Brune & Zhou, 2007). TGF-β is also known to stabilize Hif-1α via Smad3 inhibition of *Phd2* (McMahon et al, 2006). LPS in the bacterial cell wall also stimulates HIF-1α protein accumulation and mRNA activation of *HIF-1α* (Blouin et al, 2004; Frede et al, 2006). LPS can increase HIF-1α expression in a time and dose dependent manner. HIF-1α mRNA activation is provoked by LPS activation of p42/44 MAPK and NF-κB (Frede et al, 2006). It induces an active form of HIF-1 because the complex can bind HIF-1 specific DNA sequence and activates HIF-1 specific reporter under those conditions. LPS is a strong stimulator of gene expression in macrophages (Sweet & Hume, 1996). LPS has been shown to increase HIF-1α mRNA levels in a macrophage derived cell line (Blouin et al, 2004). Activation of the PI3K pathway would increase translation of already elevated mRNA leading to increase HIF-1α protein level in non-hypoxic cells (Laughner et al, 2001; Treins et al, 2002). This is in contrast with hypoxic induction of HIF-1α which relies on stabilization of Hif-1α protein. Also LPS-induced ROS generation is known to promote HIF-1α protein accumulation in macrophages (Mi et al, 2008; Nishi et al, 2008). Early in inflammation, macrophages and NLs produce a reactive oxygen species (ROS) burst which then proceeds in an uncontrolled manner and can limit antioxidants such as ascorbate which are essential for PHD function. ROS burst can therefore result in HIF-1α accumulation by limiting

PHD function (Kaelin & Ratcliffe, 2008). Cytokines such as TNF-α and IL-1β released during inflammation can further activate HIF-1α (Hellwig-Burgel et al, 1999). TNF-α activates HIF-1 by ROS, NO production, PI3K and/or NF-κB activation (Brune & Zhou, 2007).

It has been proposed that prolyl hydroxylation of IκBkinase-β and IκBα by PHD and FIH increases NF-κB activity in mice (Kaelin & Ratcliffe, 2008). NF-κB activity is required for HIF-1α protein accumulation and is a transcriptional activator of HIF-1α as well as a transcriptional factor for inflammatory cytokines (Rius et al, 2008).

1.4.2- HIF pathway and its role in inflammation

Inflammation is a complex biological response involving many different pathways and there is growing evidence linking it to the HIF activation as seen by its crosstalk with LPS and NF-κB in the section above. The mechanisms underlying this cross-talk still need to be explored. In an inflamed tissue, the oxygen concentration is often low (Sitkovsky & Lukashev, 2005) which leads to accumulation of HIF-1α protein (Albina et al, 2001; Elson et al, 2000; Hollander et al, 2001). Areas of hypoxia are present in inflamed tissues due to an infiltration and accumulation of inflammatory cells. Hypoxia is also a key feature of OM observed in *Jbo/+* and *Jf/+* middle ear. This is also why hypoxia is a physiological stimulus and a micro-environmental feature in a range of chronic inflammatory conditions (Oliver et al, 2009). Hypoxia is also a feature of bacterial infection in tissues due to increase in oxygen consumption by proliferating bacteria at the site. As a result inflammatory cells like the macrophages are likely to experience hypoxia at the site of bacterial infection and inflammation. This is why macrophages need to operate across a broad

range of oxygen tensions which could be important in modulating their behaviour in response to hypoxia. Inflammatory cells respond rapidly to hypoxia with altered gene expression, via upregulation of hypoxia inducible factors and HIF responsive genes (Burke et al, 2003; Talks et al, 2000).

1.4.3- Hypoxia regulates inflammatory cell functions

Evidence that immune cells respond to hypoxia was illustrated when it was shown that hypoxia treated macrophages are more effective at promoting angiogenesis (Knighton et al, 1983). Hypoxic macrophages express increased levels of pro-angiogenic and HIF responsive genes such as *Vegf* (Harmey et al, 1998; Lewis et al, 2000). Deletion of *Hif-1α* from myeloid cells in mice also showed a profound impairment in their inflammatory response (Cramer et al, 2003). HIF-1α protein expression has been seen in macrophage from inflamed rheumatoid synovial tissue and absent in healthy synovial tissue (Hollander et al, 2001). A number of *in vitro* studies have documented the changes in gene expression of macrophages under hypoxic conditions. Many of these genes are required for tissue revascularization, macrophage survival, recruitment, fighting infection and activation of macrophage and other inflammatory cells (Lewis & Murdoch, 2005). In fact, HIF pathways play an important role in macrophage response to bacterial infection and as described previously do not necessarily need activation by hypoxia. Apart from activating HIF-1α responsive genes, hypoxia can also activate genes independent of HIF. For example *CXCL8* expression is increased under hypoxia because of increase in intracellular H_2O_2. H_2O_2 increases binding of AP-1 to *CXCL8* promoter and therefore, increases its expression which suggests a role of AP-1 as another hypoxia regulated transcriptional factor (Hirani et al, 2001). Hypoxia also

upregulates NF-κB activating transcription factor-4 and *Egr-1* in human and murine macrophages *in vitro* (Chandel et al, 2000; Elbarghati et al, 2008; Zampetaki et al, 2004).

1.4.4- HIF pathway and innate immunity

HIF-1 activation has been observed during inflammation with upregulation of classical hypoxia induced genes such as *Vegf* and *Glut1* (Elson et al, 2000). Functional studies using conditional HIF-1$\alpha^{-/-}$ knockout in myeloid cells have also suggested a close connection between HIF-1α pathway and immunity (Cramer et al, 2003). Peritoneal macrophages and NLs isolated from myeloid HIF-1$\alpha^{-/-}$ mice have about 80% reduction in ATP levels and diminished expression of glycolytic enzymes such as Glut1 protein. LPS stimulation is known to decrease cellular ATP in macrophages which suggests a high energy demand during macrophage activation (Lall et al, 2008). *Hif-1α* $^{-/-}$ myeloid cells (monocytes, macrophages and NLs) may have a defect in energy production in macrophages and NLs which can affect their activation (Cramer et al, 2003). Deletion of Hif-1α in macrophages also attenuates iNOS induction after bacterial stimulation (Peyssonnaux et al, 2005) as iNOS promoter which contains inflammation related transcription site also contains an HRE site, making it a key hypoxia induced gene which links HIF pathway to inflammation (Melillo et al, 1995). During inflammation HIF-1 is also critically involved in i*NOS* upregulation while reduced NO production in *Hif-1α* $^{-/-}$ cells impairs macrophage function and TNF-α production (Peyssonnaux et al, 2005). HIF-1α reduces *Staphylococcus aureus* infection in mice (Zinkernagel et al, 2008). Hypoxia has also shown the ability to increase the ability of macrophages to phagocytose bacteria (Anand et al, 2007). *In vivo* and *in*

vitro studies on mouse macrophages have revealed that HIF-1α increases their phagocytic capacity. This has been associated with increased p38MAPK phosphorylation (Anand et al, 2007). NLs display a key role in innate immune response with NL recruitment being a key characteristic of initial inflammatory phase (Stewart et al, 1981). When NLs requirement is higher, apoptosis is delayed, which increases the lifespan of inflammatory NLs. Hypoxic exposure has also shown to delay NL apoptosis *in vivo* (Mecklenburgh 2002). Reduced survival was also observed in hypoxic NLs from mice with *Hif-1α* deletion in myeloid cells (Walmsley et al, 2005). This survival is dependent on NF-κB and associated with *Hif-1α* dependent increase in *NF-κB p65 & IKK-α* mRNA levels. This links HIF pathways to NF-κB. HIF dependent NF-κB activation in macrophages releases inflammatory protein-1l-1β, which protects NLs from apoptosis (Walmsley et al, 2005). This can have consequences during inflammation as uptake of apoptotic NLs results in alternative activation of macrophages and release of anti-inflammatory cytokines such as TGF-β (Mosser & Edwards, 2008). Enhanced NL survival would disturb the healing processes triggered by TGF-β. The role of Hif-1α in delaying NLs apoptosis was further supported by evidence from a germline mutation in the *Von Hippel–Lindau* gene (*vhl)* gene. As mentioned above, Vhl plays a key role in degradation of Hif-1α protein in normoxia. A germline mutation in *vhl* resulted in enhanced NLs survival (Walmsley et al, 2006). All this suggests that HIF activation is important for delaying NLs apoptosis in hypoxia.

Molecular mechanisms underlying cross talk between HIF and inflammatory pathways are complex and partially understood but studies have established a link between the two via NF-κB pathway.

Macrophages & neutrophils –The two edged sword of inflammation

Inflammation is a formidable ally in the body's battle against infection and yet a fearsome foe if left unregulated. The initial response to infection or injury is orchestrated by the innate immune system and is usually resolved within hours and first there is an influx of NLs at the site of inflammation followed by monocytes. Other cells such as eosinophils, basophils, natural killer (NK) cells are also involved in innate immunity but NLs and macrophages are the key regulators of the inflammatory response and the main phagocytic cells.

1.5.1- Neutrophils

NLs form the majority of leukocytes and are also known as polymorphonuclear leukocytes (PMNs). NLs are granulocytic and actively phagocytic cells that produce large quantities of reactive oxygen species and proteolytic enzymes which are cytotoxic to microbes and participate in inflammatory cell recruitment (Moraes et al, 2006). As they protect against bacteria, a sufficient amount of NLs is essential for clearing infection. NLs express combined markers of CD11b (receptor for the inactivated form of complement C3) and Gr-1 antigen; which allows their definitive detection by FACS (Goni et al, 2002). This is why these markers in combination were used as NLs markers for FACS throughout this thesis.

1.5.2- Macrophages

Macrophages are mononuclear phagocytes which are derived from circulating precursors of myeloid origin. These precursors are known as monocytes and are present in blood, bone marrow (BM), spleen, liver etc. Monocytes are different from macrophages in that monocytes lack

characteristic phagocytic capacity of macrophages. These circulating monocytes migrate to different tissues and receive macrophage differentiating cues from NLs or disturbed tissue resulting in their differentiation into macrophages (Laskin et al, 2011). Some macrophages become fixed macrophages in tissues whereas others are motile and free macrophages and travel via ameboid movement through tissues. Monocytes and macrophages are highly phagocytic and characterized by large cell size (11-14 μm diameter) with indented nuclei and surface expression of CD11b and F4/80(Henkel et al, 1999). Monocytes and macrophages are recruited more slowly to the site of inflammation than NLs but are essential drivers of inflammation. Chronic inflammation is characterized by prolonged persistence of macrophages at the site of inflammation. An inflammatory response is typically triggered by activation of resident macrophages by pathogen-associated molecular patterns (PAMPs) interaction with TLRs leading to release of pro-inflammatory cytokines which further sets off a cascade of release of inflammatory cell recruiting cytokines and chemokines which drive the inflammation. Acute inflammation commences with NL recruitment followed by recruitment of monocytes which differentiate into macrophages at the site of inflammation.

1.5.3- Resolution of inflammation

Acute inflammation is characterized by the increased permeability of the vascular endothelium, vasodilation and expression of adhesion molecules on endothelial cells lining the blood vessels. Vasodilation during an inflammatory response aids in recruitment of inflammatory cells to the site of inflammation by supplying more blood to the site of inflammation. These re-circulating inflammatory cells bind to the leukocyte cell adhesion

molecules (CAMs) expressed by endothelial cells enabling them to extravasate into the inflamed tissues. Resolution of inflammation requires termination of inflammatory cell recruitment and removal of recruited cells in a controlled manner through programmed cell death or apoptosis (Porcheray et al, 2005; Savill, 1997a; Savill et al, 1989; Serhan & Savill, 2005). Failure in removal of apoptotic cells may result in necrosis of those cells leading to consequences of inflammatory kind which is characterized by persistent inflammation (Fadok et al, 2001).

1.5.3.1- Role of neutrophils in resolution of inflammation

As outlined above, the inflammatory cells initially recruited to inflamed tissue are NLs. NL infiltration peaks within the first 6 h of inflammatory response, with an increased NLs production in the BM to meet the surging need (Rankin, 2010; Summers et al, 2010). Effective elimination of NLs present at the inflamed/injured tissue is a perquisite for resolution of inflammatory response (Savill, 1997a). NLs are recruited from the circulation to the site of injury or infection by acute inflammatory chemokines such as CXCL1 (also known as growth related protein α or GRO α) and CCL2 (also known as monocyte chemoattractant protein-1 or MCP-1). NLs are recruited after resident macrophage activation but they are relatively short-lived and their recruitment is followed by recruitment of monocytes during the course of an inflammatory response. Activated NLs phagocytose bacteria or apoptotic cells at the site of inflammation and undergo apoptosis to release inflammatory mediators such as CCL3 also known as macrophage inflammatory protein α (MIP-1α) (Kasama et al, 1994; Kasama et al, 1993) and chemokines such as CXCL2 (also known as MIP-2) (Bennouna et al, 2003; Chertov et al, 1997) which attract

macrophages to the site of inflammation. NLs also secrete myeloperoxidase (MPO) (Lefkowitz & Lefkowitz, 2001) which is taken up by macrophages expressing macrophage mannose receptors (MMRs) (Shepherd & Hoidal, 1990). This interaction leads to the release of macrophage secreted inflammatory mediators such as reactive oxygen species (ROS), TNF-α, IL-1, IL-6, and Granulocyte/macrophage colony stimulating factor (GM-CSF) from macrophages (Lefkowitz et al, 2000; Lefkowitz et al, 1996; Lincoln et al, 1995). Cytokines produced by macrophages also prolong NLs life span from 6-12 h to 24-48 h (Hume et al, 2002; Lee et al, 1993; Takano et al, 2009; Yamashiro et al, 2001). Activation of master regulator of inflammation, transcription factor NF-κB by the release of cytokines such as TNF-α from macrophages can slow down NLs apoptosis which can result in chronic inflammation if left unregulated (Ward et al, 1999). However, release of 'death cytokines' such as Fas by macrophages can over ride this effect and trigger NLs apoptosis in neighboring NLs (Brown & Savill, 1999). Other micro-environmental conditions such as hypoxia have also been shown to prolong NLs survival (Cramer & Johnson, 2003; Walmsley et al, 2005). Activation of the macrophages by cytokines released by NLs sequentially releases more pro-inflammatory mediators and NL and macrophage attracting chemokines to complete a vicious circle by further recruiting and activating inflammatory cells. This is why, survival of NLs if left unregulated, or failure of macrophages to clear apoptotic NLs can prolong inflammation and result in a chronic inflammatory condition.

Influx of NLs is followed by monocyte recruitment during the course of inflammation. In inflammation, programmed cell death or apoptosis especially of NLs which are predisposed to apoptosis prevents cytotoxic content of NL granules to release in surroundings. Apoptosis involves

phagocytic removal of damaged or dying cells under controlled conditions. Removal of cells from the site of inflammation is mediated via apoptosis and rapid phagocytosis after recognition of apoptotic cell associated molecular patterns (ACAMP) by phagocytes such as macrophages (Devitt & Marshall, 2011). Failure to achieve this step or a dysfunctional apoptosis or phagocytosis at the site of inflammation can result in non-resolving inflammation.

1.5.3.2- The roles of macrophages in the resolution of inflammation

As outlined above, the apoptosis of NLs causes recognition and clearance by macrophages (Savill, 1997a; Savill, 1997b; Serhan & Savill, 2005; Zhang & Mosser, 2008). In addition to clearing the necrotic or apoptotic NLs, the process of phagocytosis serves dual purpose by initiating the signal to macrophages to begin the process of exit from inflamed local tissue site to the draining lymphatics (Bellingan et al, 1996; Savill, 2001). Uptake of apoptotic cells also stimulates macrophages to release anti-inflammatory mediators such as TGF-β1 and IL-10 (Fadok et al, 1998a; Fadok et al, 1998b; Huynh et al, 2002; Lucas et al, 2003; Voll et al, 1997) which inhibit inflammatory macrophage activation (Stuart et al, 2002). If apoptotic cells are not ingested rapidly, they progress undergo necrosis and the cell debris released after necrosis contains macrophage activating proteins such as heat-shock proteins (Zhang & Mosser, 2008). These proteins and cytokines have a dramatic effect on the physiology of macrophages, facilitating a shift towards the production of pro-inflammatory cytokines. This results in augmentation of inflammation.

Macrophages also release a plethora of T-cell activators and inflammatory mediators as well as act as antigen presenting cells to T lymphocytes

(Laskin, 2009; Laskin et al, 2011). Additionally, recent publications suggest that along with driving inflammatory processes, macrophages also play an essential role in suppression of inflammation and wound repair (Laskin, 2009; Laskin et al, 2011). This suggests macrophages act as quintessential double agents- agents of defense and agents of destruction. They can both protect the host from pathogens as well as promote destructive chronic inflammation (Laskin, 2009; Laskin et al, 2011).

1.5.3.3- Different polarized states of macrophages-M1 & M2

Normally, in a resting state, macrophages are activated by a variety of stimuli in the course of an immune response. Microbial PAMPs, LPS, cytokines secreted from T helper (Th) cells (IFN-γ) and NLs, and recognition of apoptotic cell associated molecular patterns (ACAMPS) on apoptotic cells can all serve as initial activating inflammatory activation or anti-inflammatory activation) stimulus for macrophages. Macrophages are phenotypically heterogeneous and engage in activities of almost opposing nature: pro-inflammation vs. anti-inflammation (Gordon, 2003; Stout & Suttles, 2004). The activities macrophages engage in are dependent on their activating signal(s). Macrophages can be classified broadly as classically activated (M1) or alternatively activated (M2) macrophages on the basis of their function. M1 polarized macrophage populations up-regulate inflammation (Laskin, 2009; Martinez et al, 2008) while M2 polarized macrophages activate Th2 responses, down-regulate inflammation and initiate wound repair by releasing cytokines such as IL-4, IL-10 and IL-13 (Gordon, 2003). In the course of an inflammatory response, macrophages arrive within 5-6 h after the inflammation begins; these macrophages are M1 polarized, classically activated cells recruited by cytokines released by

NLs and resident macrophages. PAMPs like LPS, endogenous danger signals such as heat-shock proteins or cytokines like IFN-γ or TNF-α act as ligands for Toll-like receptors (TLRs), intracellular pattern-recognition receptors (PRRs) and interleukin-1 receptor (IL-1R) present on the macrophage cell surface (Kono & Rock, 2008). The TLR family in particular plays a critical role in PAMP ligand detection and macrophage activation (Akira et al, 2006). Many of the afore-mentioned receptors utilize adaptor molecule MyD88 to activate NF-κB (Arancibia et al, 2007) (Chen et al, 2007). Phagocytosis of apoptotic cells by macrophages triggers production of anti-inflammatory mediators such as TGF-β (Arnold et al, 2007). TGF-β is a potent suppressor of classically activated M1 macrophages (Tsunawaki et al, 1988) and a mediator of tissue repair. IL-10 is another such suppressor of M1 macrophage activation released after phagocytosis of apoptotic cells (Mosser & Edwards, 2008). Members of scavenger receptor and C-lectin families also play an important role in microbial recognition and host inflammatory response regulation (Gordon, 2002). Scavenger receptors such as SR-A, CD-36 recognize endogenous ligands such as ACAMPS and play a key role in phagocytosis of apoptotic cells (Savill et al, 2002). These receptors also mediate uptake of oxidized lipids and formation of foamy macrophages which are commonly associated with chronic inflammatory diseases such as atherosclerosis, chronic kidney disease, verruciform xanthema (Glass & Witztum, 2001; Vaziri & Norris, 2011).

Activation of M1 macrophages triggers a panoply of genes induced by a combination of transcription factors such as signal transducer and activator of transcription-1 (STAT-1), NF-κB and mitogen activated protein kinases (MAPK)(O'Shea & Murray, 2008). Classical activation of macrophages

induces polarized Th1 responses, production of ROS, reactive nitrogen species (RNS) and pro-inflammatory cytokines (IL-23, IL-12, IL-1β, TNF-α, IL-6) (Laskin et al, 2011; Mantovani et al, 2005; Mantovani et al, 2004). Activated M1 macrophages exhibit increased phagocytic activity and increased expression of class II MHC molecules. Pro-inflammatory and cytotoxic molecules are released by M1 macrophages including cytokines such as TNF-α, IL-1 and IL-6 which promote inflammation and induce apoptosis of NLs (Laskin et al, 2011). Activated macrophages also release chemokines, an important group of cytokines that recruit inflammatory cells to the site of injury. C-X-C chemokines promote NL inflammation by recruiting and activating NLs and C- C chemokines induce migration and activation of monocytes and migration of macrophages that exhibit anti-inflammatory activity. M1 macrophages also release inflammation inducing proteolytic enzymes such as matrix metalloproteinases (MMPs) which are basically enzymes involved in extracellular matrix degradation and angiogenesis (Laskin et al, 2011). ROS and RNS are also generated via enzyme catalyzed reactions by enzymes such as NADPH oxidase and NOS-2. NOS-2 is a key inflammatory enzyme that produces relatively long-lived cytotoxic oxidant peroxynitrite (Roberts et al, 2009). Expression of *Nos-2* is upregulated in M1 macrophages (Edwards et al, 2006; Laskin, 2009; Zhang & Mosser, 2008). When generated in excess quantities, ROS and RNS species cause oxidative stress which leads to activation transcription factors NF-κB and AP-1 to further augment the inflammatory response (Laskin et al, 2011).

The activity of M1 macrophages is balanced by M2 macrophages. M2 macrophages are induced by IL-4, IL-13, IL-10 and TGF-β and down regulate inflammation but also promote Th2 response. Exposure of

macrophages to TGF-β or the Th2 lymphokine IL-4 prevents polarization of macrophages into inflammatory M1 phenotype (Duffield, 2003). As described previously, inflammatory cells at the site of inflammation may die by apoptosis. Macrophages limit inflammation by rapidly taking up apoptotic cells. If left uncleared these cells would undergo necrosis to induce pro-inflammatory M1 macrophages (Duffield, 2003; Savill, 1997b). When macrophages ingest apoptotic cells, they release anti-inflammatory mediators such as IL-10, prostaglandin 2 (PGE 2) or TGF-β. M2 macrophages mediate their anti-inflammatory and angiogenic activities by further secreting IL-4, IL-10, IL-13, TGF-β, VEGF (Duffield, 2003; Martinez et al, 2006; Martinez et al, 2008). M2 macrophages show enhanced capacity of antigen presentation and uptake of cell debris and generate anti-inflammatory cytokines (Erwig et al, 1998; Goerdt et al, 1999; Stein et al, 1992). Resolution of inflammation involves release of anti-inflammatory mediators such as TGF-β, IL-10 and clearance of classically activated macrophages from site of injury which are brought into effect by alternatively activated M2 macrophages. M2 macrophages are therefore essential for resolution of inflammation and tissue repair. M2 macrophages are further sub-grouped as M2a (activated by IL-4 and IL-13), M2b (activated by immune complexes in combination with IL-1β and LPS) while M2c (activated by IL-10, TGF-β or glucocorticosteroids) (Laskin et al, 2011). M2 macrophages are important mediators of angiogenesis as well as play key role in regulating Th2 cell functioning (Laskin et al, 2011).

In summary, macrophages responding to tissue injury are activated by inflammatory signals in their microenvironment and develop into classically activated M1 macrophages which release mediators important in host defense. Phagocytosis of apoptotic cells and release of TGF-β, IL-10, IL-4

induces alternatively activated M2 macrophages which generate products that downregulate inflammation. An imbalance in the activity of these macrophage populations can lead to excessive production of pro-inflammatory mediators or subnormal anti-inflammatory mediator production causing inflammation. Differences in gene expression profiles of M1 and M2 macrophages are exemplified in gene array studies showing that IL-10 repressed 63 and further enhanced 15 of 259 genes induced by LPS in macrophages (Lang et al, 2002). Microarray expression studies have also indicated phenotypic heterogeneity of macrophages (Williams et al, 2002) (Wells et al, 2003). However, it should be noted that classification of macrophages M1 and M2 polarized states is an something of an oversimplification and macrophage populations can exhibit plasticity, heterogeneity and modulate their responses according to changing micro-environment (Martinez et al, 2008; Porcheray et al, 2005; Stout & Suttles, 2004). The same macrophage may take part in initial pro-inflammatory and cytotoxic reactions and then later participate in resolution of inflammation and wound healing (Martinez et al, 2008; Mosser & Edwards, 2008; Porcheray et al, 2005).

Dysregulation in the function of macrophages can have detrimental effects. Hyper-responsive M1 macrophages and sub-responsive M2 macrophages can cause tissue damage and persistent inflammation while overactive activation of M2 macrophages can predispose the host to infection making macrophages a double edged sword when it comes to chronic inflammation.

Table 2.2- Stimuli for classically and alternatively activated macrophages

Mϕ activation	Stimulus
Classical	IFN-γ plus pro-inflammatory cytokines
	Bacterial lipoproteins (TLRs)
	Bacterial DNA (TLRs)
	Parasitic proteins/carbohydrates (TLRs)
	Opsonized particles (FcR, CR)
	Hypoxia
	Abnormal matrix
Alternative	IL-4
	IL-10
	IL-13
	TGF-β
	Glucocorticoids

Adapted from (Duffield, 2003). Terms in parentheses represents the stimuli through which the receptors act

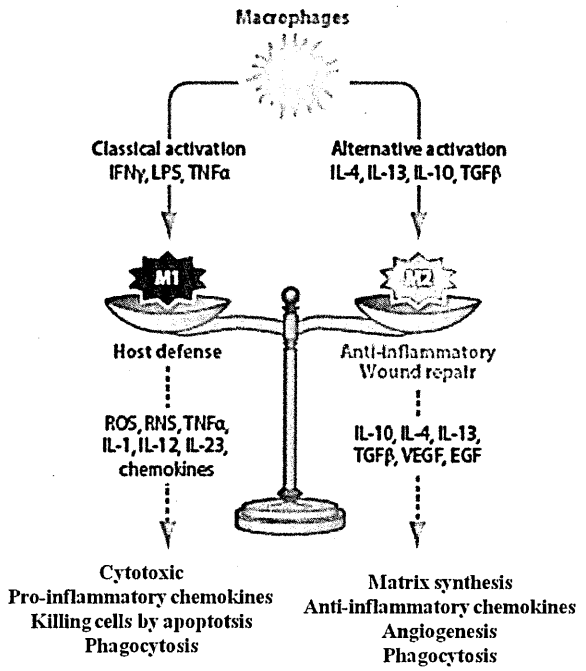


Figure 2.6- Classically and alternatively activated M1 and M2 macrophages.

Adapted from (Duffield, 2003; Laskin et al, 2011). Abbreviations: IFN-γ:Interferon-γ, IL:Interkeukin, RNS:Reactive nitrogen species, ROS:Reactive oxygen species, VEGF: Vascular endothelial growth factor

1.5.4- Foamy macrophages and inflammation

As outlined above, dysregulation of macrophages or their function can have detrimental effects on inflammatory cascade. Both M1 and M2 macrophages are capable of lipid accumulation (Peiser & Gordon, 2001; van Tits et al, 2011). Such cells are termed as foamy macrophages. Foamy macrophages are formed by uptake of oxidized low-density lipoporotein (ox-LDL) and subsequent accumulation of cholestrol esters and oxysterols which gives these macrophages a characteristic foamy appearance (Brown et al, 2000; Heinecke et al, 1991). Ox-LDL uptake by macrophages is known to affect their transcriptional profile (Ghisletti et al, 2007; Janowski et al, 1999). Foamy macrophages are known to be associated in pathogenesis of chronic inflammatory diseases such as arthesclerosis and colitis (Jozefczuk & Wozniewicz, 2011; Steinberg, 1997a; Steinberg, 1997b; Steinberg, 2009). Foamy macrophages are also associated with *Jbo/+* ear exudates (Cheeseman et al, 2011; Parkinson et al, 2006).

LDL particles are composed of cholesterol, triacylglycerides and phospholipids (Steinberg, 2002). LDL particles can undergo oxidation by various free radical generating enzymes such as lipid peroxidising enzyme lipoxygenase (LOX), Myeloperoxidase (MPO) and nitric oxide mechanism (Yoshida & Kisugi, 2010). ROS and RNS including superoxide anions, hydrogen peroxide, hydroxyl radicals and nitric oxide are produced in large quantities by activated macrophages during an acute inflammatory response and also oxidize LDL particles. Nitric oxide reacts rapidly with superoxide anion to form another cytotoxic oxidant peroxynitrite which can oxidize LDL (Roberts et al., 2009). Free radical mediated oxidation of LDL particles or retained cholesterol leads to formation of ox-LDL and oxysterols respectively (Roberts et al., 2009). Among oxidative agents

present in ox-LDL, a class of oxidized lipids, i.e., oxysterols, which are key modulators of gene expression in vascular macrophages and endothelial cells. Oxysterols are bioactive lipids which act as a regulator of lipid metabolism, inflammation and as toxic factors (Shibata & Glass, 2010). After the uptake and breakdown of Ox-LDL, most of the phospholipids and triacylglycerides are metabolized but retain oxysterols (Galkina & Ley, 2009). Oxidized lipids decompose to generate free aldehydes and ketones which can modify proteins, which is why Ox-LDL is scavenged avidly and ingested by macrophages via PRRs such as LDL receptors and scavenger receptors. LDL is internalised by receptor mediated process through pinocytosis and LDL receptors, or in the oxidized form via scavenger receptors. Expression of LDL receptors is feedback inhibited by the cholesterol, which accumulates inside the cell, via sterol regulatory element binding protein (SREBP). However, continued oxidation of LDL can cause a loss of recognition by LDL receptor and a shift in recognition by scavenger receptors which are further up-regulated by uptake of Ox-LDL(Brown & Goldstein, 1990; Yoshida et al, 1998). This increases accumulation of Ox-LDL components in macrophages and formation of foamy cells. Hypoxia increases uptake of LDL (Hulten & Levin, 2009). Hypoxia induced activation of macrophages produces reactive oxygen species which causes oxidation of LDL and cholesterol (Rydberg et al, 2004; Rydberg et al, 2003). Macrophages are oxygen sensitive and efficiently adapt to conditions of hypoxia by producing ATP from anaerobic glycolysis (Leppanen et al, 2006). Extensive glycolysis also promotes lactate accumulation which decreases pH. Acidic pH increases Ox-LDL binding to proteoglycans and scavenger receptors which also results in an increased uptake of Ox-LDL by macrophages (Leppanen et al, 2006).

Exposure to Ox-LDL and Ox-LDL uptake has been shown to alter macrophage responses. It is generally understood that foamy macrophages lose ability to elicit an inflammatory response upon stimulation. However, the literature is in disagreement on this point (Groeneweg et al, 2006; Robbesyn et al, 2004). Lower Ox-LDL and minimally oxidized LDL (mmOx-LDL) uptake concentration is thought to enhance inflammatory response of macrophages while higher concentration can repress the inflammatory response and induce apoptosis (Hundal et al, 2003; Robbesyn et al, 2004). Ox-LDL pre-exposure of granulocyte/macrophage colony stimulating factor (GM-CSF) differentiated BMDM has been shown to reduce NFκβ binding to target genes and decreases *TNF-α* and *IL-1β* mRNA and protein expression (Ohlsson et al, 1996). Pre-exposure to Ox-LDL also attenuated expression of most LPS induced genes in Human acute monocytic leukemia cell line (THP-1) derived macrophages (Mikita et al, 2001). On the other hand, an enhanced pro-inflammatory response to LPS in Ox-LDL treated BMDM has also been demonstrated (Groeneweg et al., 2006). Ox-LDL alters gene expression via ligand activated transcription factors such as Liver X receptors (LXRs). LXRs are oxysterol responsive proteins which act as nuclear transcriptional regulators. 25-hydroxycholesterol and other oxysterols bind and activate LXRs (Ghisletti et al, 2007; Janowski et al, 1999). Macrophage uptake of Ox-LDL results in increased levels of intracellular oxysterol. Activation of LXRs by oxysterol induces genes involved in cholesterol efflux such as ATP-binding cassette transporter1 *(Abca1)*. LXRs suppress LDL uptake by induction of Inducible degrader of LDL receptor (Idol) which ubiquitinates LDL receptor (Zelcer et al, 2009). LXR also forms dimers with Retinoid X receptors (RXRs) to regulate genes. Macrophages carry out several critical immune functions

such as ridding body of dead cells, mobilizing and recruiting other inflammatory cells, releasing cytokines that direct, amplify or control immune response. Altered control of these processes in foam cells would have profound effect on their ability to clear inflammation or infection.

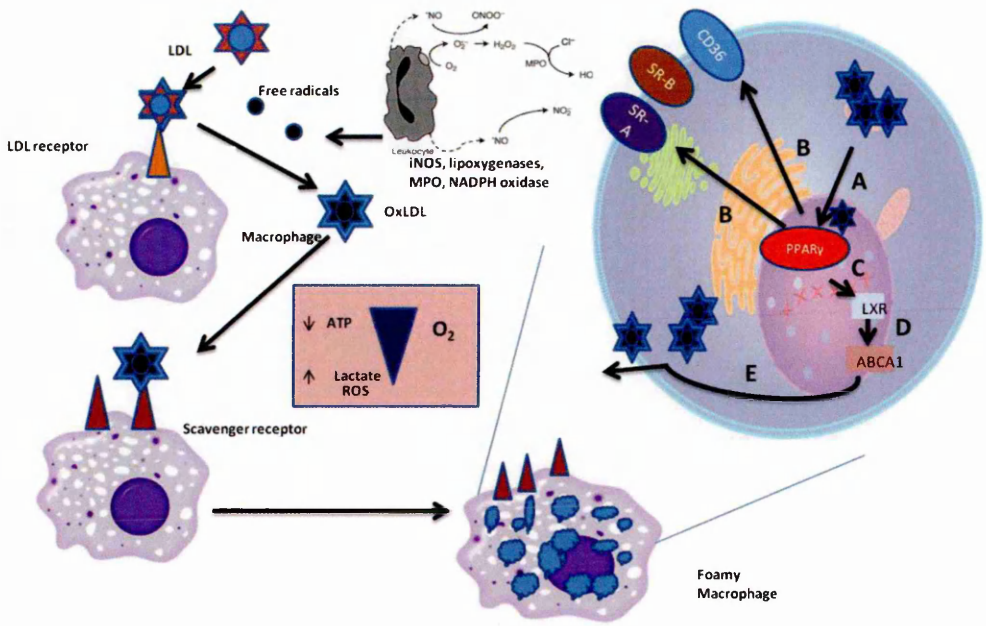


Figure 2.7- Mechanism of LDL oxidation and foamy macrophage formation.

Adapted from (Hulten & Levin, 2009; van Tits et al, 2011; Yoshida & Kisugi, 2010). LDL is oxidized by enzyme generated free radicals released by NLs and macrophages under hypoxic or activated conditions. Oxidized LDL is taken up by macrophages through scavenger receptors leading to formation of foamy macrophages. Hypoxia promotes formation of foam cells by decreasing ATP levels and increasing lactate levels to enhance Ox-LDL uptake by scavenger receptors. Foamy macrophages can modulate the by foamy macrophage under different conditions. (A) Oxysterol component of Ox-LDL inside foamy macrophages can modulate gene expression of transcription factor PPARγ which modulates gene expression of scavenger receptors (B) and activates transcription factor LXR (C) to modulate gene expression of some cholestrol efflux protein *Abca1* (D) which results in efflux of cholesterol (E).

The TGF-β Pathway

As mentioned above, chronic inflammation can be caused by excessive immune reaction or decreased immune suppression. TGF-β is a 25kD pleiotropic cytokine released by macrophages, T cells, tumour cells (Roberts, 1998) which plays an important role in immune system and wound repair (Li & Flavell, 2006; Wan & Flavell, 2007). TGF-β is also involved in angiogenesis, development, cell differentiation, cell proliferation and regulation of extracellular matrix components (ECM) (Chin et al, 1999; Dennler et al, 2002; Jeon et al, 2007; Massague et al, 1990; Shi & Massague, 2003). Most importantly, TGF-β regulates immune system by suppressing Th cell differentiation, converting naive T cells into regulatory T cells (Tregs), inhibiting production of inflammatory cytokines, suppressing pro-inflammatory genes such as *Nos2* and inducing alternative activation of anti-inflammatory M2 macrophages (Yoshimura, 2010). The importance of TGF-β in immune system regulation and the above outlined cellular processes is evident from the fact that *Tgf-β1*$^{-/-}$ knockout mice develop severe autoimmunity that leads to death within 2 wk of birth (Kulkarni & Karlsson, 1997; Kulkarni et al, 2002). TGF-β has 3 isoforms of which TGF-β1 is predominantly expressed in the mammalian immune system (Chang et al, 2002; Yoshimura et al, 2010). TGF-β is secreted as an inactive form, or latent TGF-β, which is activated by binding of latent TGF-β binding protein (LTBP) and in addition to other stimuli such as pH and ROS enable its proteolytic activation (Annes et al, 2003; Taylor, 2009). TGF-β initiates its cellular actions by binding to TGF-β serine/threonine kinase receptors (TGF-βRs). The TGF-βR family consists

of 2 groups TGF-βRI and TGF-βRII. TGF-β first binds to TGF-βRII on cell membrane; TGF-βRII then recruits and phospohrylates TGF-βRI to activate its kinase activity (Massague, 1998; Piek et al, 1999; Verrecchia & Mauviel, 2002). After ligand activation, TGF-βRI phosphorylates and activates Smad transcription factors (Massague, 2000; Massague & Chen, 2000; Massague & Wotton, 2000; Piek et al, 1999).

Smad transcription factors include receptor associated Smads (RSmads)-Smad2 and Smad3 which are recruited by TGF-βRI via Smad anchor for receptor activation (SARA) (Tsukazaki et al, 1998). These RSmads then form a heterodimer with common Smad or Smad4. This RSmad/Smad4 complex then relocates to the nucleus (Kurisaki et al, 2001; Xiao et al, 2000) where it interacts with other cofactors and acts as a transcriptional activator for target genes involved in TGF-β signalling (Massague & Chen, 2000; Massague & Wotton, 2000). TGF-β modulates expression of target genes such as *TIMP-1, PAI-1, VEGF*, which depend on Smad-interacting partners, co-activators or co-repressors (Jeon et al, 2007; Shi et al, 1998; Yoshimura, 2010). Smad proteins are essential for development and inflammation. *Smad2*$^{-/-}$ knockouts are embryonically lethal (Nomura & Li, 1998) but *Smad3*$^{-/-}$ knockouts have normal embryonic and early post-natal development but exhibit chronic inflammation and infection in different organs but have accelerated wound healing (Ashcroft et al, 1999; Yang et al, 1999). *Smad3* null mice also display increased proliferation and activation of T cells along with defects in macrophage and NL chemotaxis (Ashcroft et al, 1999; Yang et al, 1999).

In addition to the canonical Smad pathway, TGF-β signal also cross talks with other pathways such as AP-1, HIF-1 pathway, TAK1-MKK4-JNK, TAK1/MKK3.6-p38 and PI3K-Akt pathways (Derynck & Zhang, 2003;

Sanchez-Elsner et al, 2001; Yu et al, 2002; Zhang, 2009). Interestingly, there is also extensive cross-talk between HIF-1 pathway and TGF-β via Smad3 mediated inhibition of *PHD2* as well as Smad3 interaction with Hif-1α (McMahon et al, 2006; Sanchez-Elsner et al, 2001).

1.7- Concluding Remarks and Thesis Outline

The association of inflammatory gene polymorphisms with OM and the implication of host genetic factors in OM development have been demonstrated in various studies (Casselbrant et al.,1999; Kvaerner et al.,1999; 2002; Daly et al., 2004; Patel et al., 2006; Pettigrew et al., 2006, Wieresome et al., 2006; Sale & Marion 2008) (for details, see Section 1.1.2). Evidence of chronicity and high OM incidence has also been seen in certain racial demographic groups and populations with congenital and inherited syndromes. In such a scenario, it is essential to identify OM causative genetic susceptibility factors for the development of effective therapeutic measures and management of OM, especially chronic OM.

Junbo and *Jeff* mice were generated at MRC Harwell through a large-scale ENU mutagenesis program and were the first spontaneous chronic OM models. *Junbo* and *Jeff* mice carry point mutations in the genes for *Evi-1* and *Fbxo11*, respectively. Both *Jbo/+* and *Jf/+* mice display a conductive deafness phenotype (Hardisty et al, 2003; Parkinson et al, 2006). *Jbo/+* mice even develop OM even in germ-free conditions (unpublished data). However, the delay in age of OM onset in SPF facility compared to conventional facility in *Junbo* suggests that microbial load can accelerate OM progression (Parkinson et al, 2006). Pathologic hypoxia is a key feature of the middle ear in both genotypes (Cheeseman et al, 2011). Hypoxia results in the stabilisation of Hif-1α protein and activation of the HIF

signalling pathway which regulates angiogenesis and innate immune responses and is in turn regulated by inflammation at both transcriptional and translational levels (Blouin et al, 2004; Dehne & Brune, 2009; Rius et al, 2008). The mechanisms via which these mutations result in the development of spontaneous inflammation are yet to be elucidated and understood. The purpose of this thesis is to further understand and identify those mechanisms, genes and pathway that underlie chronic OM development.

It is plausible that *Evi-1*^{A2288T} mutation and *Fbxo11*^{A1472T} mutation dysregulate HIF signalling to modify the expression profile of inflammatory genes to predispose the mutants to chronic OM. In addition, both *Evi-1* and *Fbxo11* may have a role in TGF-β, signalling. (Kurokawa et al, 1998b; Tateossian et al, 2009). TGF-β is crucial for cell development, cell differentiation, M2 macrophage activation, wound repair and the inhibition of inflammatory cytokine production. Moreover, TGF-$\beta^{-/-}$ and Smad3$^{-/-}$ mice display chronic inflammation, which further suggests their crucial role in autoimmunity and inflammation. TGF-β also interacts with the HIF pathway and modulates the expression of target genes such as *Timp-1, Pai-1* and *Vegf,* which also depend on Smad interacting partners such as Evi-1 protein for expression (Derynck & Zhang, 2003; Sanchez-Elsner et al, 2001; Yu et al, 2002; Zhang, 2009). Hence, a hypothesis was formed that the mutations in *Jbo/+* and *Jf/+* may impact TGF-β signalling and result in chronic inflammation (Figure 1.8).

Extensive cross-talk exists between TGF-β, HIF pathways and inflammatory signalling pathways via their interaction with NF-κB and AP-1 transcriptional activators. Nasopharyngeal commensal bacteria also accelerate OM onset in *Jbo/+* mice. PAMPs from bacteria trigger

inflammatory signalling pathways such as the NF-κB pathway. The dysregulated or abrogated HIF or TGF-β pathway can have a profound effect on NF-κB. *Jbo/+ Evi-1*^{A2288T} mutation might also affect its interaction with AP-1, as their interaction with the HIF pathway.

The aim of this thesis is to understand how the *Evi-1*^{A2288T} mutation and *Fbxo11*^{A1472T} mutation might affect HIF, TGF-β pathways and effect innate immune system regulation. The main focus of this thesis is the impact of the mutation on HIF signalling and TGF-β signalling in *Jbo/+* mice. *Jf/+* mice were also studied. However, *Jeff* studies were limited to key experiments examining TGF-β due to time limitations. The principle approach was to use an *in vitro* system that used bone marrow derived macrophages (BMDM). NLs and foamy macrophages characterise *Jbo/+* ear exudates but NLs are difficult to work with, as they have a short life span (~6 h). *In vivo* many of the NLs have undergone apoptosis. In contrast macrophages have a longer life span. Macrophages release NL-attracting chemokines and are key to initiating and resolving inflammation. The number of macrophages recoverable from the middle ear of *Jbo/+* is too low to study in isolation. This is why an *in vivo* to *in vitro* model system was developed using BMDM. BMDM provides a large number of defined cell phenotypes, which could be challenged or activated by treatments lasting up to 72 h as the hypoxia in middle ear must be prevalent over long duration. Both hypoxia and TGF-β signalling has been studied extensively using a BMDM *in vitro* model system. The use of BMDM in vitro model has enabled the researchers to build up the complexity using a combination of hypoxia, TGF-β, LPS and oxysterol challenges to investigate chronic inflammatory conditions such as atherosclerosis, In this thesis have used this system to

model the complex micro-environment of the inflamed middle ear in *Jbo/+* and *Jf/+* mice.

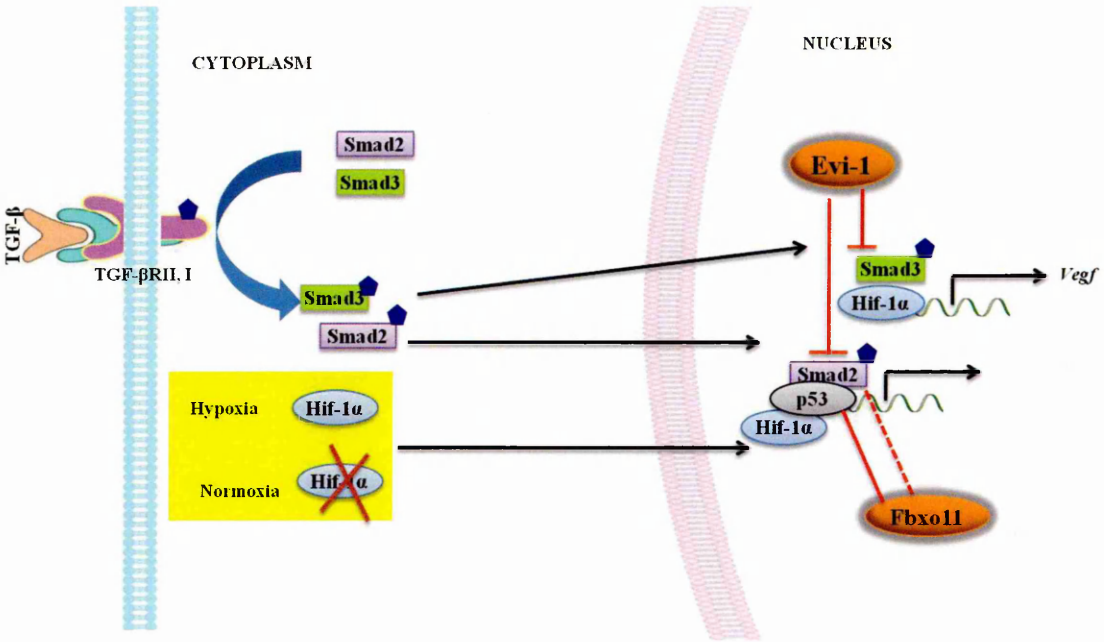


Figure 2.8- Diagrammatic representation of hypothesis

Evi-1^{A2288T} mutation in *Jbo/+* mice dysregulates TGF-β and HIF signalling
Fbxo11^{A1472T} mutation in *Jf/+* mice dysregulates TGF- β signalling via p53 and Smad2

Chapter 2

3. Materials and methods

2.1- Animal Husbandry

The founder mice carrying the *Jbo/+* and *Jf/+* mutation were generated in a large-scale ENU mutagenesis program at Harwell, United Kingdom (Hardisty-Hughes et al, 2006; Hardisty et al, 2003; Parkinson et al, 2006). *Jbo/+* mice are now congenic on C3H/HeH background. *Jeff* mice are maintained on a mixed C57BL/6J C3H/HeH background. *Junbo* and *Jeff* mice were maintained in a high-health-status specific pathogen free (SPF) unit in which all FELASA-listed pathogens have been excluded. Mice were housed in individually ventilated racks (Techniplast UK Ltd.) containing grade 6 sawdust bedding with exposure to a 12 hour light/dark cycle at 21±2°C and 55±10% relative humidity. Mice were supplied with irradiated mouse diet (Special Diets Services, UK) and water. All animal procedures were conducted according to the appropriate Home Office Project and Personal licenses and under the guidance issued by the Medical Research Council in 'Responsibility in the Use of Animals for Medical Research' (July 2003).

2.2- Genotyping

2.2.1-Genotyping using pyrosequencing

Original genotyping in the beginning of this project was carried out from DNA extracted from ear clips using Qiagen DNeasy Blood & Tissue Kit as per manufacturer's instructions. Ear clips were incubated at 56° C overnight after addition of 180 µl lysis buffer and 20µl proteinase K. Final DNA elution was performed using 100 µl elution buffer. PCR (Figure 2.1) was

performed and SNP based detection of *Jbo/+* mutation on exon 9 of *Evi-1* was detected by pyrosequencing. Pyrosequencing reaction involves a standard PCR using a biotinylated forward primer, a normal reverse primer and DNA extracted from ear clips. The internal sequencing primer, purified PCR products and Qiagen pyrosequencing reaction mix were used to run the pyrosequencing reaction using Qiagen Biotage PSQ HS 96A pyrosequencing machine as per manufacturer's instructions.

Pyrosequencing primers for *Jbo/+* sequencing

Reverse primer	5' TTTCCCATAACACCACTGAGG 3'	Biomers.net
Forward primer	5' TGTTTGCAACACTGTGTCTGTATT3'	Biomers.net

PCR was performed as a 20µl reaction containing Qiagen PCR master mix, primers (See appendix Table 8.1) and ultra pure water. 5 ng of DNA was used as template; ultra pure water was used as a control. All work was performed in ultraviolet cabinets and filtered tips were used throughout the PCR procedure.

Pyrosequencing primers for *Jbo/+* sequencing

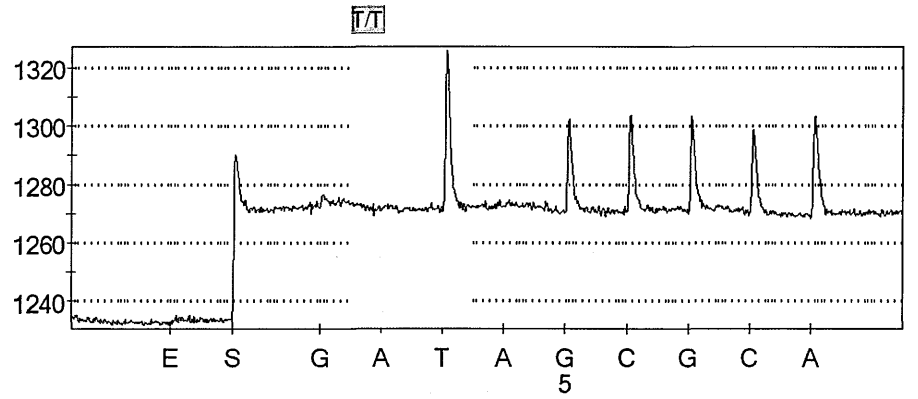
Revers primer	5' TTTCCCATAACACCACTGAGG 3'	Biomers.net
Forward primer	5' TGTTTGCAACACTGTGTCTGTATT3'	Biomers.net

The cycling profile was as follows:

Step 1	95 °C for 5 min
Step 2	95 °C for 15 s (44 cycles)
Step3	52 °C for 30 s (44 cycles)

Step 4	72 °C for 15 s (44 cycles)
Step 5	76 °C for 5 min

WT : T/T (Passed)



Jbo/+: A/T (Passed)

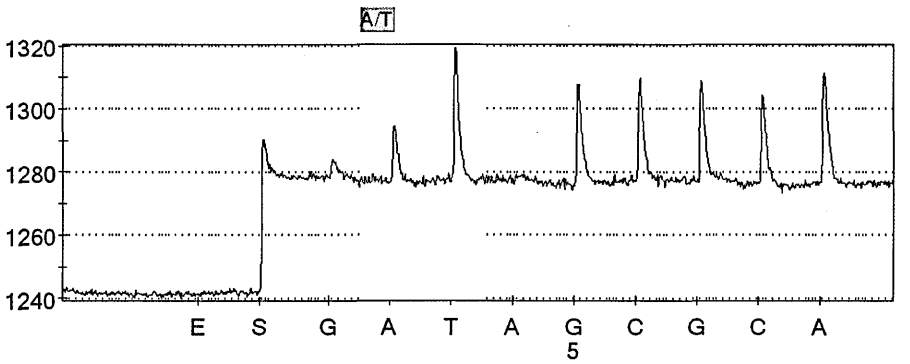


Figure 3.1- Genotyping Result Pattern for *Jbo*/+ and WT mice in Pyrosequencing

Although I performed the initial genotyping; the genotyping service was taken over by GEMS core service at MRC using the upgraded lightscanner technology (Figure 2.2) as the colony was being managed at a much larger scale for a number of projects.

2.2.2- *Jeff* and *Junbo* genotyping using Lightscanner

Genotyping of *Jeff* and *Junbo* mice was performed by GEMS core using LunaProbes on Lightscanner machine, Idaho technology. PCR was performed in the presence of dsDNA binding dye LC green under the following conditions:

***Junbo* sequencing**

*Junbo*Evi1ex11F	5'ACTGTGATAGATCATTCAGCATTTC 3'
*Junbo*Evi1ex11R	5' CTTCAGGTGTCTGTCAAGATT 3'
*Junbo*ProbeR	5' GCTTCTCCTTGTTGTGGATGATGCG 3'

PCR mix

HotShot master mix	5µl
LCGreen	1µl
*Junbo*Evi1ex11F (20ng/µl)	0.5µl
*Junbo*Evi1ex11R (20ng/µl)	0.1µl
*Junbo*ProbeR (20ng/µl)	0.5µl
DNA (1/10 dil ABI)	2µl
ddH2O	0.9µl

***Jeff* sequencing**

Primers/Probe sets 5'>3'

Fbox11*Jeff*-F	5' ACACTACTAGGTTCTAAACACTGACT 3'
Fbox11*Jeff*-R	5' TAAATCCCACCATGCTGTCCATC 3'
Fbox11*Jeff*-PR	5' CTGTTTGTCCTGATCAGAATTCCTGCTA 3'

PCR mix

HotShot master mix	5µl
LCGreen	1µl
Fbox11*Jeff*F (20ng/µl)	0.5µl
Fbox11*Jeff*R (20ng/µl)	0.1µl
Fbox11*Jeff*PrR (20ng/µl)	0.5µl
DNA (1/10 dil ABI)	2µl
ddH2O	0.9µl

PCR program for both the genotypes was as follows:

1) 95°C for 2 min
2) 95 °C for 30 s (PCR cycle)
3) 60 °C for 30 s
4) 72 °C for 30 s
5) Cycle, step 2 to 4 55 times
6) 95 °C for 30 s Hybridization
7) 25 °C for 30 s
8) 15 °C for 30 s

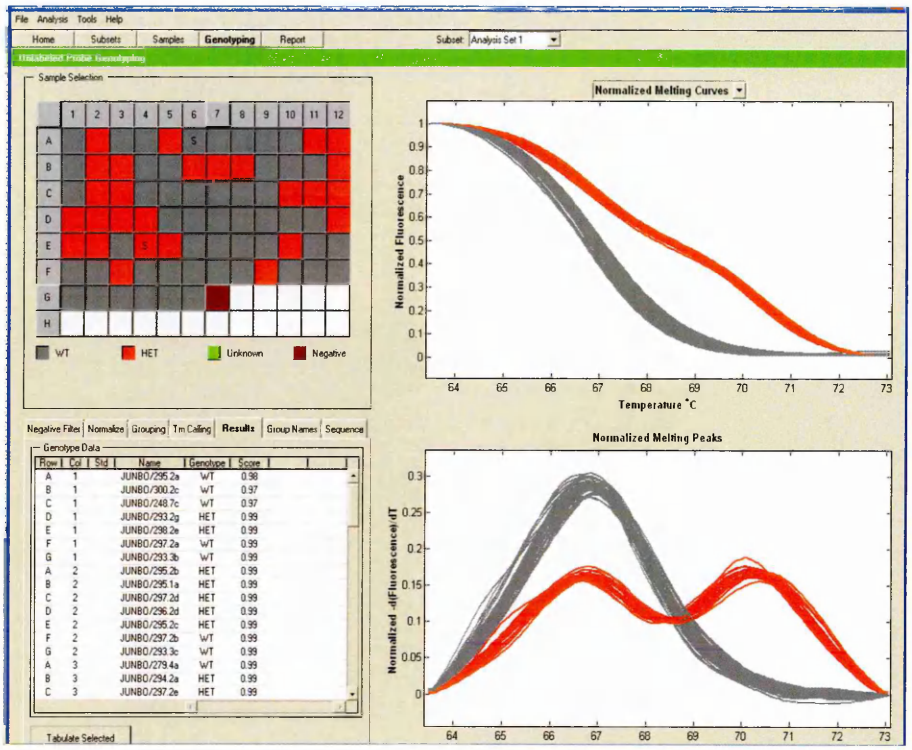


Figure 3.2- Example of lightscanner output for *Junbo* genotyping

The red samples with 2 peaks denote *Jbo/+* while the grey samples with single peak denote WT. The plate in the diagram denotes the layout with all the samples. (Image courtesy- GEMS).

2.3- Cell culture

Plastic T25 (25 cm^2) and T75 (75 cm^2) flasks for cell culture were purchased from Greiner. All tissue culture and cell culture work was performed in tissue culture room facility in a bio safety cabinet (class II). Roswell Park Memorial Institute medium (RPMI) medium and Hank's Balanced Salt Solution (HBSS) used in NL isolation were obtained from Gibco. All tissue culture experiments used Dulbecco modified eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), G418 or Gentamycin antibiotic, 1% Pencillin/Streptomycin and 1% L-Glutamine (Sigma Aldrich). DMEM 6046, percoll and brewer's thioglycollate medium was supplied by Sigma Aldrich. All media and phosphate buffered saline

(PBS) solutions were pre-warmed at 37°C for 10 min in a water bath before use. A Beckman centrifuge was used at 160 *g* for pelleting cells during cell culture. Light microscopy was performed on cells using a Nikon TS 100 F microscope (Nikon).

2.3.1- Raw 264.7 cell line

Raw 264.7 cell line is a mouse leukaemic monocyte macrophage cell line which was obtained from American Type Culture Collection (ATCC). Raw 264.7 cells were cultured in DMEM media (containing 10% FCS and 1% L-Glutamine/Pencillin/Streptomycin).

2.3.2- Granulocyte and macrophage colony stimulating factor (GM-CSF) expressing cell line

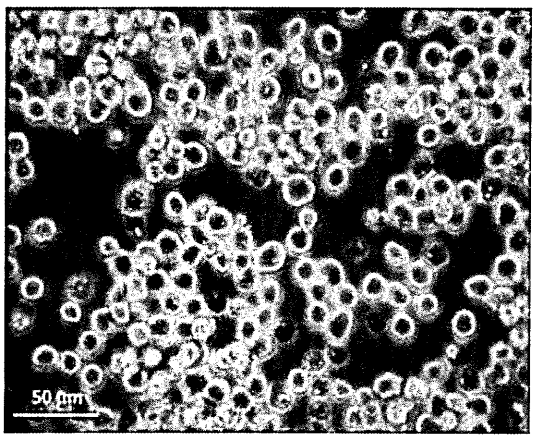
The Granulocyte and Macrophage Colony Stimulating Factor (GM-CSF) cell line is a plasmacytoma line X63-AG8 transfected with mammalian expression vector BCMGSNeo with a mouse gene for GM-CSF (Karasuyama et al, 1990; Zal et al, 1994). Frozen cell aliquots were thawed and cells were grown in selection medium (G418 at 1mg/ml). GM-CSF cells were grown up in a T175 tissue culture flask up to medium density, harvested and washed twice in medium without G418 and then returned to culture for 2-3 d in DMEM medium without G418. The cells were grown for 7 d until the cells were 65% confluent (Figure 2.3 a). The supernatant containing GM-CSF was then harvested after centrifugation at 160 *g* for 5 min twice. GM-CSF was then frozen in aliquots and stored at -80°C. GM-CSF was tested for macrophage differentiation by a series of dilutions (negative control, 1%, 2.5%, 5%, 10%) using wildtype (WT) BMDM every -time before freezing a stock.

2.3.3-BM derived macrophage (BMDM) Culture

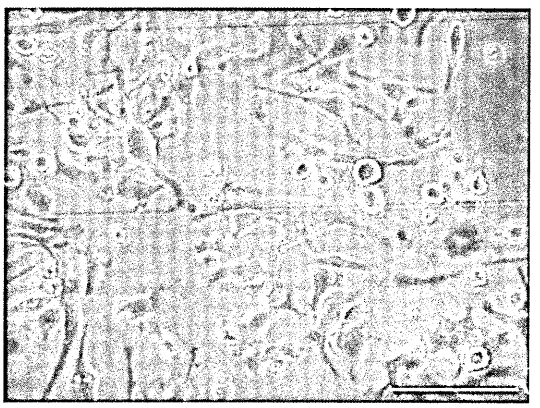
Mice were euthanized by cervical dislocation. Mouse femurs and tibias were excised, cleaned and bone marrow (BM) was flushed out using a syringe and DMEM media (containing 10% FCS and 10% GM-CSF). Cells were counted prior to seeding using haemocytometer. Cell count per flask ranged between 1 to 2 x 10^6 cells/ ml at the time of seeding. The cells were then grown in T 75 flasks at 37°C for 3 d. On the third day non-adherent cells were washed off and fresh DMEM media containing 10% FCS and 10% GM-CSF supernatant was added to the flask (Figure 2.3 b). Media was replaced with fresh medium after 6 d to yield mature macrophages from 7 d onwards. Macrophages were adherent to the plastic and were checked for purity using FACS.

2.3.3.1- BMDM Macrophage purity assay

BMDM cells grown and differentiated for 7 d using 10% GM-CSF were analyzed for purity with macrophage cell surface marker CD11b and F4/80. Cells were washed with FACS buffer at and pelleted at 530 *g* prior to use. Phycoerythrin Rat anti-mouse CD11b and F4/80 were purchased from BD Pharmingen and Gr-1 antibody from BD Pharmingen was used for NL detection. The purity assay revealed a macrophage purity of 84-87% in cultured BMDM cells.



a)



b)

Figure 3.3 - GM-CSF cell line and *Junbo* BMDM

Olympus CK2 inverted microscope and Nikon Coolpix 955 were used to take images of (a) GM-CSF cell line (Scale-bar denotes 50 µm) and (b) BMDM from WT *Junbo* grown for 7 d (Scale-bar denotes 100 µm).

2.3.5-Isolation and culture of BM derived murine neutrophil leukocytes

6-8 wk old mice of mixed gender were euthanised and BM was flushed from the femur and tibia of mice using 5 ml RPMI media. The sample was the centrifuged at 160 *g* for 6 min at 4°C. The pellet was then resuspended in 2ml 1X HBSS and placed over a 6ml 52%/64%/72% percoll gradient. The discontinuous percoll gradient was prepared using isotonic percoll (9

parts neat percoll: 1 part 10X PBS) and 1X HBSS. The gradient with the suspended cells was then centrifuged at 275 *g* for 30 min at 4°C and using a sterile glass 5 ml pipette purified neutrophil leukocytes (NL) were removed from the interface between 64% and 72% fractions (Haslett et al, 1985). The purified NLs were then cultured in T25 tissue culture flasks and incubated at 37°C.

2.3.6-Normoxic treatment

Normoxic control cultures were maintained for all experiments in an incubator with an atmosphere of 5% CO_2, and 95% filtered air at 37°C.

2.3.7-Hypoxic treatment

After seven days growth and differentiation under normoxic conditions, tissue culture flasks containing BMDM cells were transferred to mini Macs Anaerobic workstation (Don Whitley Scientific) which was flushed 1%O_2, 5% CO_2, and 94% N_2 at 37°C (gas mixture supplied by BOC). To induce hypoxia in cell cultures rapidly, hypoxia conditioned medium (24 h hypoxia conditioning in the incubator) was added to the tissue culture flasks after decanting the original media.

2.3.8-TGF-β Treatment

TGF-β1 (T7039-2 UG) was obtained from Sigma Aldrich. A stock solution of 2μg/ml was made by dissolving the TGF-β1 in 1 ml medium then aliquoted (hereafter referred to as TGF-β). The aliquots were stored at -20 °C until use. A concentration of 200 pmol was used in tissue culture media. The concentration used was optimized after a standard dosage experiment.

2.3.9-LPS Treatment

Lipopolysacharide (LPS) (L4641, Sigma Aldrich) was used as a standard. 1 mg/ml stocks were aliquoted and stored at -20 °C. A standard dose of 250 ng/ml of LPS in tissue culture media was used throughout for the LPS studies. The optimum dose was decided after a dosage experiment on WT BMDM (0, 10, 100, 250, 500, 1000 ng/ml) (See chapter 4 for details).

2.3.10-Oxysterol Treatment

Oxysterol stock was prepared by dissolving oxysterol C27, H4 O225-OH-chol or 25-diol (H1015, Sigma) in 95% ethanol to prepare a 5 mg/ml stock solution. Oxysterol used was added at a 5 μg/ml working concentration such that the final concentration of ethanol in culture medium was 0.1%. BMDM were incubated with oxysterol for 24 h after 7 d of culture in GM-CSF to induce foamy macrophage formation. 24 h oxysterol treatment in normoxia allowed uptake of oxysterol before hypoxia treatment. This incubation was followed by another 12 h with oxysterol under hypoxia or normoxia in cell media equilibrated to hypoxia or normoxia respectively. In some experiments, oxysterol was washed out for 2 h under hypoxia followed by activation with LPS (250 ng/ml). In control cells, only the ethanol vehicle was added.

2.3.11-Harvesting cells

Growth media was removed from culture flasks and the flasks were washed twice with PBS. Cells were detached using a cell scraper (Corning) into fresh PBS before being added to a 15 ml conical tube and centrifuged at 160 *g* for 5 min. PBS was removed and the pellets were frozen on dry ice before storage at -80 °C.

2.4- RNA Extraction

Harvested BMDM pellets were stored at -80 °C and were thawed on wet ice before extraction of RNA. RNA was isolated using RNeasy Plus mini kit from Qiagen according to manufacturer's instructions in hypoxia studies and TGF-β normoxia studies. As a standard procedure 600 µl of lysis buffer RLT plus (RNeasy Plus mini kit, Qiagen) was added to lyse the cells. QIAshredder from Qiagen was used to homogenize the lysate. RNA was finally eluted in 50 µl of RNase free water. Later on Nucleospin kits were adopted for RNA extraction as it allowed for extraction of RNA and protein at the same time. Extractions were conducted using a Nucleospin® RNA/protein kit (Macherey-Nagel) following manufacturer's instructions for all the other RNA studies (TGF-β hypoxia studies, LPS studies, oxysterol study) in this thesis. RNA was eluted in 60 µl RNase-free water and concentrations were determined using the Nanodrop 8000 (Thermo Scientific) and 1.4% agarose gel. Samples were stored at -20 °C.

2.5-cDNA synthesis

The isolated RNA was reverse transcribed to produce cDNA using High-Capacity cDNA Reverse Transcription Kit containing MultiScribe ™ Reverse Transcriptase (Applied Biosystems). The Kit uses random primer scheme for initiating cDNA synthesis. Reaction volume of 30 µl was used for the cDNA synthesis. 15 µl of master mix was used per reaction. An equal volume of 1 µg RNA was made up using RNase free water per reaction for each sample to be used as a template.

The following volumes were used per reaction:

Component	Volume µl/Reaction
10X RT Buffer	3 µl
25X dNTP mix (100mMol)	1.2 µl
10X Random Primers	3 µl
Multiscribe Reverse Transciptase	1.5 µl
Nuclease free water	6.3 µl

The following programme was used for the cDNA synthesis.

	Step 1	Step 2	Step 3	Step 4
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 min	Hold

cDNA samples were either used immediately in RT-qPCR reactions or stored long term at -20°C.

2.6- Pre-amplification PCR

Applied Biosystems TaqMan pre-amplification mastermix kit was used to detect low levels of *Evi-1* in BMDM. *Ppia* was used as endogenous control and a comparison of standard RT-qPCR and preamplified RT-qPCR was performed for *Tnf-α, Il-1β, Il-6* on LPS treated cells and *Phd2* on hypoxic cells to remove possibility of amplification bias.

Equal volumes of TaqMan assays were pooled for up to 100 assays and a pre-amplification reaction performed in PCR tubes using the following volumes.

TaqMan PreAmp Master Mix	25 µl
Pooled assay mix (0.2X, each assay)	12.5 µl
33.33 ng cDNA sample + nuclease free water	12.5 µl

A pre-amplification PCR for 10 cycles was then performed using the following conditions:

Step 1	95 °C for 10 min
Step 2	95 °C for 15 s
Step3	60 °C for 4 min
Step 4	54 °C holding time

The pre-amplified cDNA was then diluted 1:5 in Tris-EDTA (TE) buffer (pH 8.0). The final diluted pre-amplified cDNA was used as template for standard RT-qPCR reactions for *Evi-1*.

2.7-TaqMan ™ RT-qPCR (Real Time quantitative Polymerase Chain Reaction)

Levels of gene expression can be determined by measuring amount of cellular RNA. RT-qPCR is a powerful tool to quantify gene expression. The quantitative end point of PCR cycle is threshold cycle or C_T which is defined as the PCR cycle at which fluorescent signal of reporter dye crosses an arbitrarily placed threshold (Schmittgen & Livak, 2008a). C_T is inversely proportional to the amount of amplicon in the reaction. So lower the C_T, greater the amount of amplicon. C_T values are logarithmic and are used either directly (comparative C_T method) or indirectly (interpolation to standard curves to create linear values) for quantifying relative gene expression.

cDNA prepared as a 30 µl volume was diluted 4.5 µl in 115.5 µl in nuclease free water in Non stick Rnase free 1.5 ml microfuge tubes (Ambion). TaqMan Fast Universal PCR master mix (2 X) from Applied Biosystems was used as a mastermix. 20X TaqMan assay corresponding to each gene was used (Appendix 8.1). *Ppia* was used as an endogenous control for all

experiments except the Oxysterol studies. *Gapdh* was used as the endogenous control for Oxysterol studies as *Ppia* C_T Cycle changed by >6 folds with oxysterol treatment.

Each reaction required 4 µl (5ng) of cDNA; subsequently a master mix was prepared as per manufacturer's instructions with 5ng cDNA per reaction. Reaction volume of 16 µl was pipetted carefully using Eppendorf combitips, to avoid bubbles, into a 0.1 ml Fast Optical 96 well reaction plate (Applied Biosystems) and centrifuged for 1 min at 160 *g* before being loaded into the ABI 7500 Fast qPCR System (Applied Biosystems). Three technical replicates were performed for each sample and each probe along with non template control. The plate was sealed with MicroAmp Optical Adhesive Film (Applied Biosystems) and loaded into the RT-qPCR machine (Applied Biosystems). The assay was run as arelative plate quantification (RQ) programme.

Components	**1 well/µl**
Fast Master Mix	10
20× TaqMan assay	1
cDNA (5ng)	4
water	5

2.7.1-Standard curve

The standard curve (Figure 2.4) method involves constructing a standard curve from RNA of a known concentration; which is then used as a reference for extrapolating quantitative information of mRNA targets of unknown concentrations (Livak & Schmittgen, 2001). This method requires the least amount of validation but is only useful for a small number of

samples and low numbers of gene targets. A standard curve was performed to check efficiency of TaqMan probes.

For relative quantification to work the efficiency of PCR reaction must be between 90 and 110% efficient. To calculate this, real time PCR was performed on a dilution series of cDNA (0.1 ng/µl, 1 ng/µl, 10 ng/µl, 100 ng/µl) and a standard curve was generated from ΔC_T results (Described in next section). Reactions were carried out in triplicate for each concentration with a non template control (NTC). Efficiency of probe was calculated by $10^{(-1/x)}-1$ into 100 where x is the slope value efficient. Efficiency of all probes was calculated to be between 90-110%. The efficiency of all TaqMan probes is claimed to be ~ 100% by the manufacturer.

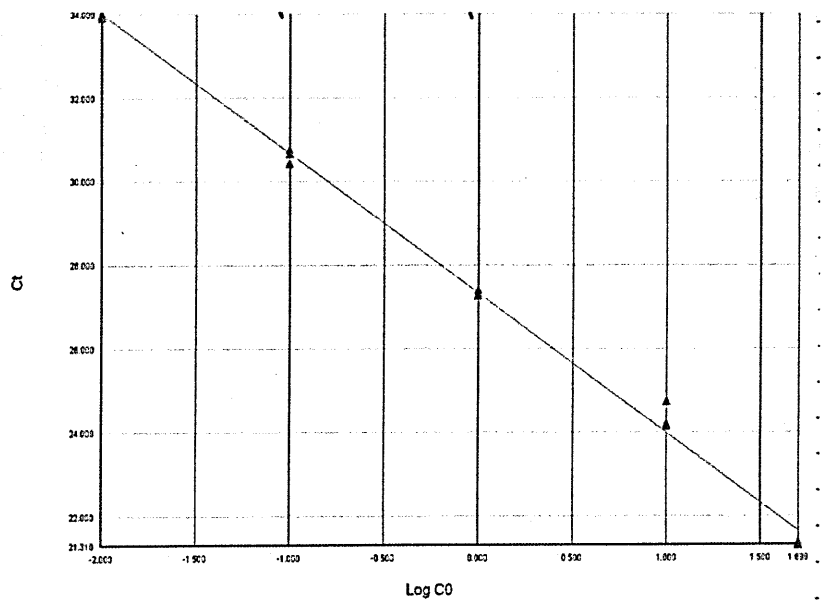


Figure 3.4-A representative standard curve for TaqMan probe for assay for Hif-1α

The slope was calculated and used to calculate efficiency (For e.g., the slope for Hif-1α standard curve is -3.1999 and the efficiency = 99.1%.

2.7.2- Relative quantification

Another method of presenting RT-qPCR is by comparative C_T method oralso known as $2^{-\Delta\Delta C}{}_T$ method. It is a gold standard method for interpreting

RT-qPCR data and widely used for its ease of use. The comparative C_T method involves comparing the C_T values of the samples of interest with a calibrator simple. The C_T values of both the calibrator and the samples of interest are normalized to an appropriate endogenous housekeeping gene. For the $\Delta\Delta C_T$ calculation (see below for description) to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal.

Fold change or RQ is obtained from the final form of $2^{-\Delta\Delta C}{}_T$ equation, the derivation of which has been reported in Applied Biosystems User Bulletin No 2 (P/N 4303859) and (Schmittgen & Livak, 2008b)

Fold change or RQ = $2^{-\Delta\Delta C}{}_T$

Where $\Delta\Delta C_T = \Delta C_T$ (target sample) – ΔC_T (calibrator sample)

This allows for data to be interpreted as expression of gene interest relative to the internal endogenous control in target to be compared to calibrator as fold change.

Throughout this thesis, RT-qPCR results were normalized to the levels of *Ppia* or *Gapdh* RNA and relative quantification was calculated using $2^{-\Delta\Delta C}{}_T$ as recommended by Applied Biosystems. Relative mRNA expression was expressed as fold expression over the calibrator sample, the average gene expression corresponding to the appropriate calibrator control group. Statistics were then performed on $2^{-\Delta\Delta C}{}_T$ or $2^{-\Delta C}{}_T$ normalized individual data points to further confirm difference in gene expression level between genotypes (Schmittgen & Livak, 2008b).

2.8-Statistics

2.8.1- Statistics for RT-qPCR

Statistics were performed using RQmin-max as recommended by Applied Biosystems (ABI 7500 Fast Realtime Software). Data was expressed as bar graphs in which error bars display RQmin and RQmax (upper and lower limits defining region of expression within which the expression levels fall) for graphical representations of basal gene differences between genotypes. The data was plotted as bar graph.

A true fold difference for each experiment was calculated using formula $2^{(\text{Maximum Ct x} - \text{Minimum Ct x})}$ for each given experiment where x is the endogenous gene control. As a general rule a fold difference ≥ 2 fold was accepted as a true difference.

For each biological sample, standard deviation of ΔC_T value was calculated. RQmin and RQmax were calculated using formula $RQmin = 2^{-[\text{average } \Delta\Delta CT + (SEdCT*t)]}$ and $RQmax = 2^{-[\text{average } \Delta\Delta CT - (SE\Delta CT*t)]}$ where SE is standard error and t is the t value from a student t-test table for a given degree of freedom at $\geq 95\%$ confidence. Bar graph values where 95% confidence limit error bars did not overlap were deemed significant. *P* values of ≤ 0.05 were considered significant.

2.8.2- Other analysis and statistics

Ingenuity Pathway Analysis Software was used to diagrammatically represent genetic interactions (www.ingenuity.com).

Student's t test was used for analysis of experiments other than RT-qPCR (See above). Prism Statistical Analysis Software 5 was used to do statistical analysis and graphically represent the data. Standard Error of Mean (SEM)

was defined by error bars in each experiment. *P* values of ≤0.05 were considered significant.

2.9- Protein extraction

For hypoxia studies cytoplasmic and nuclear protein extraction was carried out using Paris Kit from Ambion as per manufacturer's instructions. The nuclear and cytoplasmic lysates were then pooled together to make a total cell lysate. Protease inhibitor (P8340 from Sigma Aldrich) was added to the lysis buffer (1000X) before use.

For the LPS studies, the Nucleospin extraction kit was used to obtain protein pellets as per manufacturer's instructions. Protein precipitation was performed by washing the pellet in ice cold acetone then centrifugation at 13, 201 *g* for 5 min. The tubes were then dried at 30 °C. The pellet was then re-suspended by pipetting in 50 µl of NP40 lysis buffer (150 mM NaCl, 1% NP-40, 50mM Tris ph 8.0, supplemented with protease inhibitors, phosphatase inhibitors).

2.10- Protein quantification

Protein concentrations in lysates were determined using the Bradford assay. Bio-Rad's Quick Start Serum Albumin Standard Test was used to generate a standard curve in each experiment as per manufacturer's instructions in the 250 µl microplate assay format. A BMG Labtech Optima plate reader was used to measure the absorbance at 595 nm.

2.11-Western Blotting

2.11.1- Western blotting for Hif-1α

Electrophoresis of 35 µg of protein lysates from BMDM was performed using Bio-rad's 7.5% Tris HCl pre-cast gels. The gel was run for 1 h at 110

V using Mini Protean 3 Cell by Bio-rad. Gel was transferred on nitrocellulose membrane using wet transfer method at 100 V for 1 h with ice pack. The membrane was blocked with 5% non fat dry milk in Tris buffered saline with x% Tween 20 (TBST) for 1 h at room temperature. The blot was then incubated either with antibodies against Hif-1α (NB100-479 by Novus Biologics), MPO (AF3667 by R & D Systems) or β-actin (Ab8227 from Abcam) overnight at 4 °C. The membrane was then incubated with goat polyclonal to rabbit IgG (Abcam, ab7090) for Hif-1α and β-actin and rabbit polyclonal to goat IgG (Abcam, ab6741) for MPO at a concentration of 1:2000 for 1 h at room temperature. A chemiluminescent signal was developed on Kodak XOmat AR Film by using Pierce Supersignal West Pico detection system. Hypoxia treated Raw 264.7 cells were used as a control.

2.11.2- Western blotting for Evi-1

Samples were run on Invitrogen's NuPAGE Novex Bis-Tris gels (4-12%) as per manufacturer's instructions. The gel was run in X Cell *Sure Lock* Mini Cell (Invitrogen) for 50 min at 200V using 3-(N-morpholino) propanesulfonic acid (MOPS) running buffer.

Mini iBlot dry blotting system by Invitrogen was used for transferring the gel onto nitrocellulose membrane using iBlot transfer device. The transfer was performed at 20 V for 8 min.

The membrane was then blocked using 2.5% non fat dry milk in 1 X PBS with 0.1% Tween 20 detergent for 1 h. Primary antibody for Evi-1 86306 (supplier) was added at a concentration of 1:1000 or for β- actin (1:1000) in blocking buffer to the membrane and incubated overnight. The blot was washed three times for 10 min with PBS 0.1% Tween. Anti-rabbit antibody

was added at a concentration of 1: 10,000. The blot was washed again three times for 5 min each in PBS with 0.1% Tween.

Amersham ECL advance western blotting detection kit was used for signal detection on Amersham X Ray film. Thermo Restore™ Western Blot Stripping Buffer was used for stripping the membrane for 10 min at 37°C wherever re-probing with another antibody was required. The membrane was subjected to a blocking treatment prior to another primary antibody treatment.

2.12- Proteome Profiler Array

For determination of relative mouse cytokines and chemokine levels, an antibody based cytokine array-Proteome Profiler Array (ARY006, R & D Biosystems) was used.

The supernatant from the treated BMDM T75 flasks was pooled and aliquoted into 1.5 ml tubes and frozen at -80°C. BMDM cells were washed in ice-cold PBS and harvested. The cells were pelleted at 160 *g* for 5 min. PBS was then aspirated and the cell pellet was soubilized in 1 ml lysis buffer (1% Igepal CA-630, 20mM Tris-HCl ph 8.0, 137mM NaCl, 10% glycerol, 2mM EDTA). 10 µg/ml each of protease inhibitors Aprotinin (A6279, Sigma Aldrich), Leupeptin (L8511, Sigma Aldrich), Pepstatin (P4265, Sigma Aldrich) were added to the lysis buffer just before use. The cell pellet was gently re-suspended by pipetting and gently rocked at 4°C for 30 min. The lysate was then centrifuged at 13, 201 *g* for 5 min. The supernatant was transferred on a clean tube and protein in the lysate was quantified using Bradford assay. The cytokine array detection was then performed on cell lysates (122µg of protein) and supernatants (600µl) as per manufacturer's instructions.

Signal detection was achieved using Supersignal West Pico Chemi-luminescent detection system by Pierce ansd X-Ray film Kodak Biomax™ Light-1 (Kodak). Signal quantification via densitometry was performed using Vision Works LS Software version 6.8 by UVP and Chemidoc-It Imaging system (UVP).

2.13-Fluorescence activated cell sorter (FACS)

2.13.1- Thioglycollate elicitation of macrophages and NLs

To assess recruitment of inflammatory cells mice were injected intraperitoneally with oxidized 4% Brewer's thioglycollate solution (Sigma Aldrich) according to body weight (10 ml/kg). At 1 d or 4 d after thioglycollate treatment mice were euthanised by cervical dislocation, and cells were recovered by peritoneal lavage using 5 ml of cold DMEM. DMEM was aspirated back into the syringe, transferred to a 15 ml tube and stored on wet ice. Cells were counted and analysed using an ADVIA haematology analyser (Siemens) as well used for FACS analysis using BD FACS Canto II.

For FACS analysis cells were stained with Allophycocyanin (APC) conjugated anti–mouse F4/80 (MF48005, Caltac Labrotaries), FITC conjugated anti-CD11b (557396, BD Pharmingen) and PerCP-Cy 5.5 anti-mouse Gr-1 (552093, BD Pharmingen). Cells were added to a 96 well plate and fixed with lysis buffer before incubation with the antibodies. A dilution of 1:400 was used for CD11b and F4/80 while a dilution of 1:1600 was used for Gr-1.

2.13.2- Neutrophil leukocyte apoptosis assay

BM derived NLs were harvested and incubated in normoxic or hypoxic conditions for 20 h. Apoptosis and cell necrosis was assessed by FACS with

fluorescein isocynate (FITC) labeled Annexin V and Propidium Iodide (PI) staining using a Apoptosis Detection Kit by BD Pharmingen as per manufacturer's instructions.

2.13.3- Lox-1 assay

Lox-1/SR-E1-Phycoerythrin was obtained from R & D Systems. Lox-1 expression on BMDM was assayed by FACS.

2.13.4- Ox-LDL Assay

Serum samples were collected from *Jbo/+* and WT littermate controls and stored at -80°C. Middle ear fluid from the same cohort of *Jbo/+* mice was collected in 100µl of ice-cold PBS. The samples were mixed gently and centrifuged at 5000 *g* for 20s at 8°C. Supernatant was aliquoted into 95µl batches and stored at -80°C until assay. Ox-LDL in the samples was then measured using the mouse ox-LDL ELISA kit (Uscn Life Science Inc.).

Chapter 3

4. Immune cell function in *Junbo*

3.1- Introduction

Many OM models exhibit a continuum of pleiotropic effects of mutation which result in systemic auto-immune defects and chronic OM. Examples include *MyD88*$^{-/-}$ mice (Rye et al, 2011a), *lpr/lpr* mice (Rivkin et al, 2005) amongst others. Heterozygous *Junbo* mice (*Jbo/+*) develop spontaneous OM. *Jbo/+* mice develop spontaneous inflammation even in germ-free conditions (unpublished results) and it is important to consider whether the chronic middle ear inflammation is a result of systemic defects in immune cell recruitment and inflammation resolution. My hypothesis is that OM in *Jbo/+* mice is a result of dysregulated response of inflammatory cells to specific micro-environmental conditions unique to the *Jbo/+* middle ear which augments the inflammation and prolongs its persistence.

NLs and macrophages are the innate immune cells which determine host's ability to ward off infection and resolve inflammation. NLs are the first inflammatory cells to be recruited to the site of inflammation followed by macrophages. Defects in systemic recruitment of NLs and macrophages can result in susceptibility to inflammation or infection. Resolution of inflammation requires both appropriate cell recruitment and subsequent removal of recruited cells in a controlled manner. Both processes are essential for proper clearance of inflammation. For instance, low circulating NL numbers, neutropenia, can render a patient susceptible to microbial infection whilst chronic inflammation is characterized by a continued influx

of NLs into the inflamed site (Sadik et al, 2011). Infiltrating NLs play a tissue damaging role in a number of chronic conditions such as chronic obstructive pulmonary disease (COPD), asthma, and rheumatoid arthritis usually due to dysregulation of their recruitment in tissues (Drost et al, 2005; Edwards & Hallett, 1997; Hogg, 2004; Monteseirin, 2009). These infiltrating neutrophils release toxic oxygen intermediates and proteases, which can result in tissue damage (Movat, 1979)

Monocyte recruitment follows NLs recruitment during the course of inflammation and on activation release important NL attracting CXC chemokines (Hume et al, 2002; Lee et al, 1993). A higher number of recruited and activated macrophages would result in increased influx of NLs resulting in further tissue damage if the inflammation is not resolved. In inflammation, apoptosis is a crucial cellular process which requires phagocytic removal of damaged or necrotic cells from the site of inflammation. This process is mediated via apoptosis and rapid phagocytosis primarily by macrophages. Macrophages ingest apoptotic NLs and a reduced macrophage recruitment would result in delayed clearance of apoptotic NLs resulting in secondary necrosis. Debris released after necrosis is loaded with macrophage activating endogenous factors, such as heat-shock proteins (Zhang & Mosser, 2008). These proteins and cytokines facilitate a shift towards pro-inflammatory cytokine production by macrophages and other NLs which would exacerbate inflammation.

No differences have been observed in the numbers and maturity of NLs in blood between WT and *Jbo*/+ mice (Parkinson et al, 2006). However, NL and macrophage recruitment has not been studied.

In this chapter, I have investigated the presence of Hif-1α signal in the ear-exudate from the middle ear of *Jbo*/+ mice. Ear exudates from *Jbo*/+ mice

contain large numbers of hypoxic viable, apoptotic and necrotic NLs as well as hypoxic foamy macrophages (Parkinson et al, 2006). Hypoxia stabilizes Hif-1α and evidence for this in *Jbo/+* ear exudates was sought by Hif-1α western blotting. Evi-1 protein expression was also checked in the ear exudates obtained from *Jbo/+* mice using western blotting.

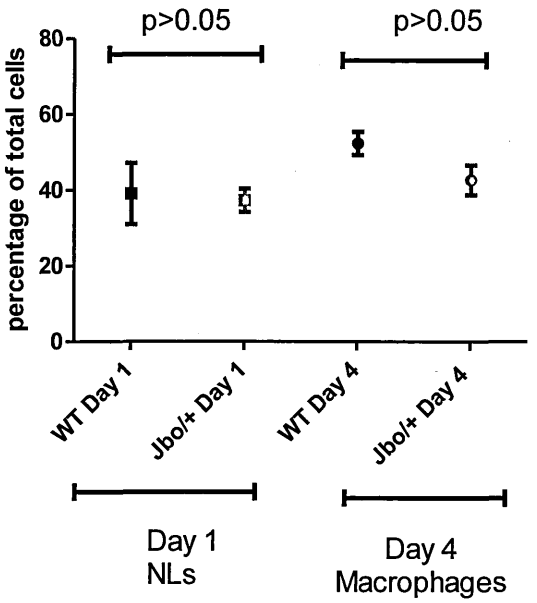
One of the regulating factors of inflammation is the ability of NLs to undergo apoptosis (Haslett, 1992) which triggers clearance of apoptotic NLs by macrophages, initiating M2 polarization of macrophages and inducing wound-repair (Laskin et al, 2011; Savill, 1997a; Savill, 1997b; Savill et al, 1989; Serhan & Savill, 2005). Intrinsic apoptotic threshold of NLs can be modified by a range of external stimuli such as cytokines and oxidative stress. Hypoxia has also been shown to prolong NL survival (Cramer & Johnson, 2003; Walmsley et al, 2005). Studies on anoxia challenge of murine bone marrow derived NLs (BMDNLs) from Hif-1$\alpha^{-/-}$ mice and human NLs show that hypoxia inhibits NL apoptosis and a role of HIF pathway in regulation of NL apoptosis via MIP-1β, NF-κB and HIF-1α (Walmsley et al, 2005). Delayed NL apoptosis can in turn release more pro-inflammatory mediators and cytokines that amplify further recruitment and activation of inflammatory cells. NLs have a short life-span of ~6–8 h in humans and ~11 h in mice (Rankin, 2010; Summers et al, 2010) which makes them difficult to utilize in gene and protein expression studies which involved treatments lasting up to 72 h. This is why macrophages were made the focus of study in this thesis henceforth. Macrophages play a major role in resolution of inflammation, initiate NL influx by releasing NL attracting chemokines and are also found in exudates from *Jbo/+* mice. As the number of macrophages recovered from the middle ear of *Jbo/+* mice is too low to study in isolation, all gene expression studies from this chapter onwards

were performed on BMDM. This is a system which also allows for addition of further levels of complications to mimic the complex micro-environment of inflamed middle ear.

3.2- Results

3.2.1- Immune cell recruitment in *Jbo/+* is not dysregulated in a standard intra-peritoneal challenge model

One of the first experiments performed was to investigate NL and macrophage recruitment in *Junbo* mice using a well-established model of inflammation; an intra-peritoneal injection of thioglycollate. Thioglycollate broth has been used extensively to study kinetics of inflammation (Leijh et al, 1984; Melnicoff et al, 1989). Peritoneal injection of thioglycollate elicits an inflammatory response resulting in the recruitment of NLs after 1 day and macrophages after 3-4 days (Leijh et al, 1984; Melnicoff et al, 1989; Qureshi & Jakschik, 1988). NLs were detected by FACS using combined markers of CD11b and Gr-1 antigen which allows their definitive detection (Goni et al, 2002). Macrophages were detected by using surface markers CD11b and F4/80(Henkel et al, 1999). FACS analysis of inflammatory cells elicited by thioglycollate challenge in *Jbo/+* and WT mice was performed. No differences were observed between *Jbo/+* and WT mice in neutrophil recruitment, macrophage recruitment or white blood cell (WBC) numbers. This suggests that the *Evi-1* mutation does not have a global effect on inflammation in the peritoneal inflammation model in *Junbo* (Figure 3.1).



Recruitment of NLs and macrophages after thioglycollate

Figure 4.1- Intra-peritoneal thioglycollate injection reveals no global defects in inflammatory cell recruitment in *Jbo/+* mice

Thioglycollate was injected in *Jbo/+* mice intra-peritoneally as described in Chapter 2. Peritoneal fluid was collected and analysed using FACS. Majority of cells collected after day 1 were Gr-1 and CD11b positive NLs. The majority of cells collected at day 4 were Gr-1 and F4/80 positive macrophages. Closed blue squares/circles represent WT NLs/ macrophages and closed red squares/circles represent *Jbo/+* NLs /macrophages. Error bars denote SEM. Statistics was performed on arc-sin values of percentage using student's t-test. P >0.05. n=6.

3.2.2- Stabilization of Hif-1α in the middle ear in *Jbo/+* mice

Western blotting for Hif-1α was performed on ear exudates (Figure 3.2). As ear exudate from *Junbo* mice contains a mixture of NLs and macrophages, BMDNLs were used as negative control for hypoxia. Myeloperoxidase (MPO) is a peroxidase enzyme present in neutrophil granules. MPO and β-actin were used as a loading control for this experiment. Ear exudates from five *Jbo/+* mice were collected in 150 µl cell lysis buffer and protein extraction was carried out using Paris kit by Ambion as per manufacturer's instructions.Total cell lysate (nuclear lysate + cytoplasmic lysate) and western-blotted for Hif-1α. As there is no exudate present in WT mice, BMDNLs were used as a negative control. A strong Hif-1α signal was observed in *Junbo* ear exudates, albeit at a lower band size of ~88-90kD as compared to the expected 110 kD band size. (Explanation in section 3.3). However, a Hif-1α signal was not observed in the negative control normoxic BMDNLs. Stabilization of Hif-1α is consistent with the demonstration of hypoxia *in vivo* using histo-chemical pimonidazole labelling (Cheeseman et al, 2011).

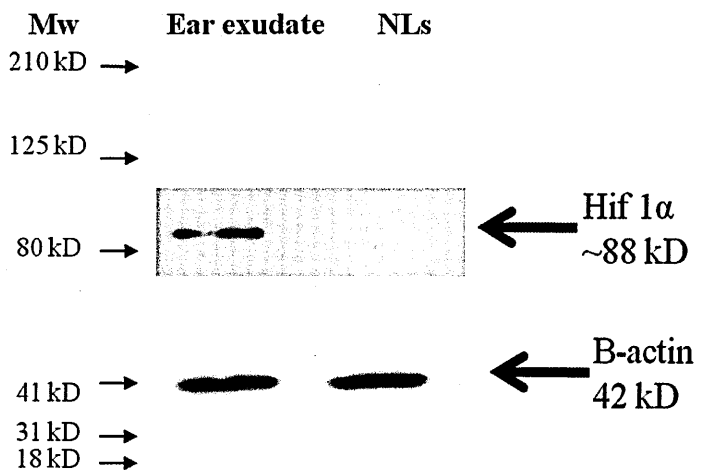


Figure 4.2- Ear exudates in *Jbo/+* mice is hypoxic

35 µg of total cell lysate from ear exudates were prepared and blotted for Hif-1α and β-actin using Hif-1α antibody NB100-479 and β-actin antibody Ab8227 respectively.

3.2.3- Evi-1 is expressed in *Jbo/+* ear exudates

Total cell lysate obtained from ear exudates was blotted for Evi-1 (Figure 3.3) and a signal was observed in ear exudates. Kidney lysates were used as a positive control as kidney cells are known to express the *Evi-1* gene (Morishita et al, 1990b). This was an independent confirmation that Evi-1 is expressed in *Jbo/+* middle ear which suggests that the Evi-1 mutation does not affect the protein expression. However, there was no suitable WT counterpart for measuring the relative levels between the genotypes is not available as WT mice do not have ear-exudate.

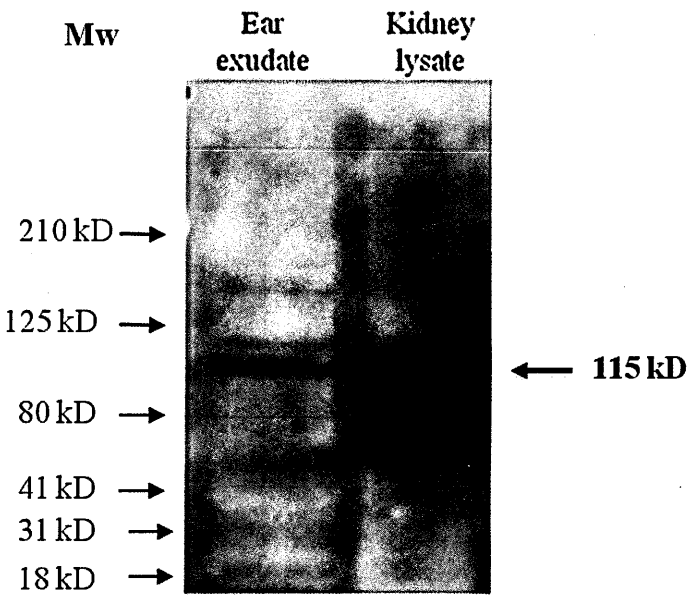


Figure 4.3- Evi-1 protein is expressed in *Jbo/+* ear-exudate

20 µg of total cell lysates from pooled ear-exudate *Jbo/+* ear-exudate from 6 individual 6-8wk old mice was western blotted for Evi-1 (1:1000). Total kidney lysates (3 µl) was used as a positive control as *Evi-1* is highly expressed in kidneys. Evi-1 band was observed at 115-120 kD.

3.2.4- Neutrophil apoptosis is not dysregulated in *Jbo/+* mice

To observe if NL apoptosis in *Jbo/+* mice is delayed or dysregulated compared to WT mice, a series of NLs apoptosis studies on *Junbo* BMDNLs was performed using FACS. BMDNLs were used to study NL apoptosis in hypoxia in *Junbo* mice as inflamed middle ear in *Jbo/+* is hypoxic (Cheeseman et al, 2011). This approach was chosen because WT *Junbo* mice do not have ear fluids to harvest NLs as controls and NLs from *Jbo/+* ear fluid exudates are a mixture of viable, apoptotic and necrotic cells and are relatively few in number. Apoptosis is identified by a specific chain of morphological changes occurring in dying cells. Externalization of phosphotidylserine (PS) is an early event in apoptosis and occurs whilst cellular membrane is still intact. Annexin V specifically binds to PS and hence detects early stages of apoptosis (van Engeland et al, 1998). In late apoptotic or necrotic cell, loss of cell membrane's structural integrity occurs and results in uptake of membrane impermeable dyes such as propidium iodide which allows for detection of necrosis and latter stages of apoptosis (McCarthy & Evan, 1998). To quantify apoptosis in *Jbo/+* and WT BMDNLs, NLs were isolated from bone marrow of mice and exposed to 20 h normoxia or hypoxia to study effect of hypoxia on NL apoptosis. Gr1-staining for NLs, Annexin V staining for apototic cells and PI (propidium iodide) staining for cell necrosis and late apoptosis was performed and analysed using FACS. No difference in early or late apoptosis stages was observed between *Jbo/+* and WT BMDNLs (Figure 3.4).

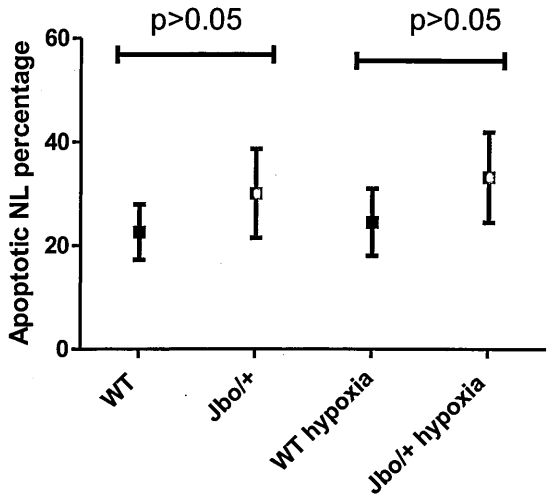


Figure 4.4- NL apoptosis is comparable in WT and *Jbo/+* BMDM in conditions of normoxia and hypoxia

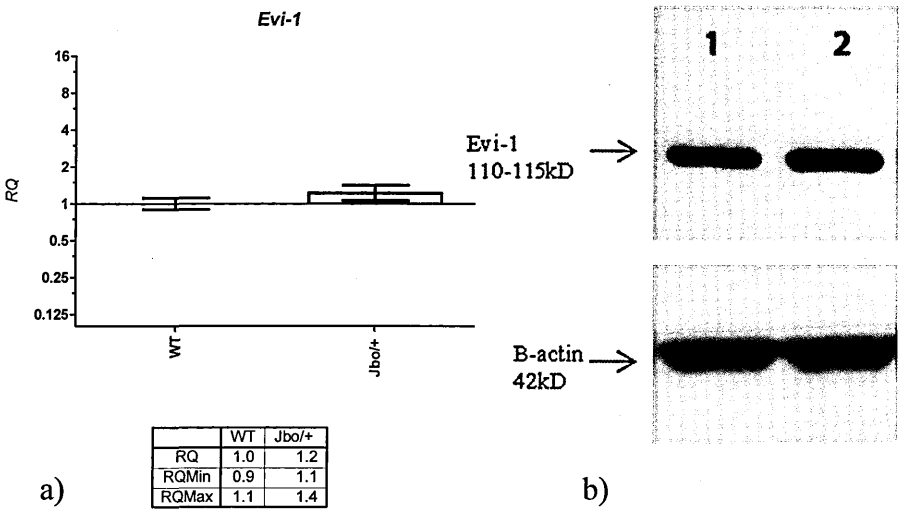
BMDNLs were treated to 20 h hypoxia or normoxia. FACS was then performed for Annexin V which binds to phosphotidylserine (PS) externalized on apoptosis. Closed blue squares represent WT and closed red squares represent *Jbo/+* mice. Error bars denote SEM. Statistics was performed on arc-sin values of percentage of apoptotic cells using student's t-test. Genotypic differences were not considered significant as $P > 0.05$. N=5 for WT BMDNLs; N=7 for *Jbo/+* BMDNLs; N= 6 for WT and *Jbo/+* hypoxia treated BMDNLs.

3.2.5- *Evi-1* is expressed in *Junbo* BMDM

RT-qPCR assays for *Evi-1* were performed on BMDM differentiated with GM-CSF for 8 d using preamplification PCR as *Evi-1* levels were at borderline detection level with standard RT-qPCR assay. *Ppia* was used as an endogenous control for the assay. The results indicate that *Evi-1* was expressed in both *Jbo/+* and WT BMDM in normoxic controls albeit at low levels (Figure 3.5a).

A strong Evi-1 protein signal was detected in both *Jbo/+* and WT BMDM in unstimulated and normoxic conditions with western blotting using total cell lysate extracted from 8 d old (Figure 3.5b).

Evi-1 expression has been observed in myeloid and epithelial cells of middle ears without any genotypic differences between WT and *Jbo/+* (Parkinson et al, 2006). This result is of importance because it suggested that the *Evi-1*^{A2288T} mutation in *Jbo/+* does not affect the expression of Evi-1.



	WT	Jbo/+
RQ	1.0	1.2
RQMin	0.9	1.1
RQMax	1.1	1.4

a) b)

Figure 4.5- *Evi-1* gene and protein is expressed in WT and *Jbo/+* BMDM at comparable levels

a) Relative *Evi-1* expression was comparable in both WT and *Jbo/+* resting BMDM. Pre-amplification PCR was performed prior to RT-qPCR due to high average cycle threshold of *Evi-1* (>32). Statistics were performed using RQ min-max as recommended by Applied Biosystems using the ABI 7500 sds software. N= 3.
b) 20 μg of total cell lysates from WT and *Jbo/+* BMDM were western blotted for Evi-1 (1:1000) and β actin (1:10,000). Evi-1 is expressed in both WT (Lane 1) and *Jbo/+* BMDM (Lane 2) at comparable levels. Blot was qyantified using Vision Works LS Software v 6.8 (UVP).

3.2.6- Resting WT and *Jbo/+* BMDM have comparable levels of Evi-1 responsive, hypoxia responsive and TGF-β responsive genes in normoxia

Next, basal gene expression levels (RQ *Jbo/+* / RQ WT) of *Smad3, Hif-1α, Abca1* (a key cholesterol transport controlling gene) as well as Evi-1 responsive genes (*Gata2, Map3k14, Jun, Fos*), hypoxia and TGF-β responsive genes (*Vegf, Glut-1, Phd2, Pai-1*) and an inflammatory gene panel (*Il-1β, Il-6, Ccl2, Cxcl2, Tnf-α, Nos-2*) were compared between WT and *Jbo/+* BMDM using RT-qPCR. My hypothesis is that HIF signalling and TGF-β signalling is dysregulated in *Jbo/+* BMDM due to the *Evi-1*^{A2288T} mutation. A significant difference ($P < 0.05$) in gene expression levels between the two genotypes at basal level of Evi-1 responsive genes *Gata2, Map3k14, Jun,* and *Fos* as well as *Ccl2,* the gene encoding for NL and monocyte chemoattractant Ccl2 was observed. However the difference in gene expression levels observed was at all cases less than the 2 fold cut-off threshold level which is the generally accepted as the minimum difference that is likely to be biologically robust. *Ppia* was used as an endogenous control. No significant differences between expression of *Vegf, Glut-1, Phd2, Pai-, Il-1β, Il-6, Cxcl2, Tnf-α* and *Nos-2* was observed between *Jbo/+* and WT mice (Figure 3.6).

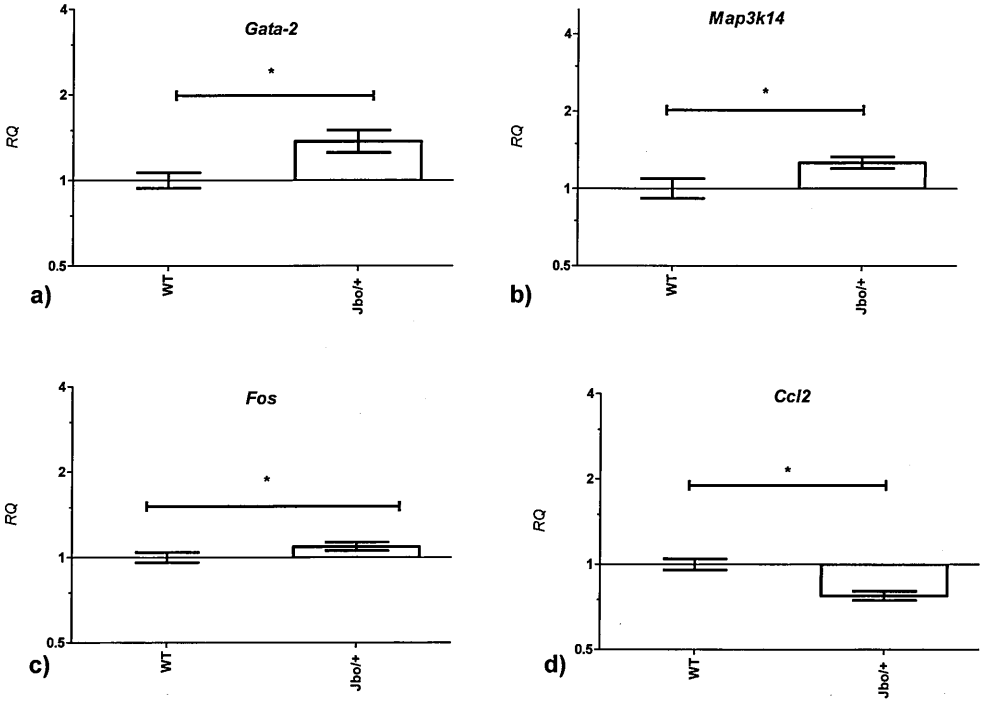


Figure 4.6- Comparative gene expression levels of Evi-1 responsive, hypoxia responsive and pro-inflammatory genes

This figure illustrates relative gene expression levels between WT and *Jbo/+*resting BMDM in normoxia. Three biological replicates were used for this study. Each replicate was obtained from 8d old BMDM from a pool of 5-6 individual mice (6-8 wk old). Statistics were performed using RQ min-max as recommended by Applied Biosystems using the ABI 7500 sds software. Blue bars denote WT levels and red bars denote *Jbo/+* levels. *Jbo/+* were calibrated to WT. The error bars represent RQ min and RQ max. Gene expression data obtained was normalized to *Ppia* gene expression. * $P < 0.05$. Figure panel shows difference in relative levels of (a) *Gata-2* (b) *Map3k14* (c) *Fos* (d) *Ccl2*

Table 4.1- Difference in gene expression level between WT and *Jbo/+* in resting condition

Gene	WT	*Jbo/+*
Abca1	1.0 (0.7, 1.4)	1.0 (0.7, 1.4)
Ccl2	**1.0** (1.0, 1.0)	**0.8** (0.7, 0.8)
Cxcl2	1.0 (1.0, 1.0)	1.0 (1.0, 1.1)
Fos	**1.0** (1.0, 1.0)	**1.1** (1.1, 1.1)
Gata-2	**1.0** (0.9, 1.1)	**1.4** (1.3, 1.5)
Glut-1	1.0 (1.0, 1.0)	0.9 (0.8, 1.0)
Il-1β	1.0 (0.8, 1.2)	1.0 (0.8, 1.1)
Il-6	1.0 (0.9, 1.1)	0.8 (0.7, 0.9)
Jun	1.0 (0.9, 1.1)	1.2 (1.1, 1.3)
Map3k14	**1.0** (0.9, 1.1)	**1.3** (1.2, 1.3)
Nos-2	1.0 (1.0, 1.0)	1.0 (0.9, 1.0)
Pai-1	1.0 (0.5, 2.1)	0.9 (0.5, 1.6)
Phd2	1.0 (0.9, 1.1)	1.1 (1.1, 1.1)
Smad3	1.0 (0.8, 1.2)	0.8 (0.7, 0.9)
Tnf-α	1.0 (1.0, 1.0)	1.0 (0.9, 1.0)
Vegf	1.0 (0.9, 1.1)	1.0 (0.9, 1.0)

Table 3.1 shows the difference in gene expression level between WT and *Jbo/+* BMDM at resting state. Values in parenthesis represent RQmin-max values. Coloured numerical values (blue) represent significant difference between genotypes ($P < 0.05$). However, numerical figures in blue were not considered biologically relevant as they did not make a cut-off threshold of 2 fold up/downregulation.

3.3- Discussion

No difference in NL or macrophage recruitment was observed in the thioglycollate model of inflammation which suggests that *Jbo/+* mice do not have a global defect in the inflammatory response. This indicates that the *Evi-1*^{A2288T} mutation in *Jbo/+* mice does not affect systemic recruitment of inflammatory cells or the resolution of inflammation so must have a more specific role in OM. This brings environmental factors such as hypoxia to the forefront of my subsequent investigations of chronic OM observed in *Junbo*.

In vivo labelling of middle ear in *Junbo* with hypoxia marker pimonidazole has previously revealed the site to be hypoxic and filled with ear exudate composed of hypoxic populations of NLs and foamy macrophages (Cheeseman et al., 2011). Hypoxia commonly occurs at sites of inflammation and the unmet oxygen demand of large numbers of NLs in ear exudates may drive this process. Under normoxic conditions, Hif-1α is degraded after hydroxylation of its prolyl residues by PHD2 (Masson et al., 2001; Lando et al., 2002). However, in hypoxic conditions stabilization of Hif-1α occurs. Hence, anti-Hif-1α is often used as a protein marker for hypoxia. Western blotting of ear exudates with anti-Hif-1α displayed a strong signal albeit at a lower size of 88-90 kD. This may be due to partial proteolysis by neutrophil elastases and proteases. A strong MPO signal was also observed in ear exudates which confirmed that large number of NLs is present in ear-exudate. The demonstration of Hif-1α stabilization is consistent with result obtained by Cheeseman et al. (2011) that the inflamed middle ear of *Jbo/+* mice middle ear is hypoxic.

Evi-1protein was detected in ear exudates from the *Jbo/+* middle ear. This result was confirmed in BMDM when low but constant levels of *Evi-1* gene

expression were observed in both WT and *Jbo/+* BMDM, accompanied with high levels of protein expression in both genotypes. These results suggest that the mutation in *Evi-1* in *Jbo/+* mice does not interfere with transcription or translation of the protein. Future work into the half-life of Evi-1 and its binding to other proteins with pull down assays will throw more light on how *Evi-1* mutation might affect Evi-1 binding in *Jbo/+* mice. As mentioned before, ear-exudate from *Jbo/+* middle ear is rich in NLs. In such a scenario, understanding NLs apoptosis and necrosis is critical because it can potentially alter their phagocytic uptake by macrophages and result in release of pro-inflammatory cytokines in the middle ear of *Jbo/+* mice. Results showed no difference in NL apoptosis between *Jbo/+* and WT mice under hypoxic or normoxic conditions. Contrary, to the observation by Walmsley et al. (2005) that hypoxia exerts a protective mechanism on human NLs we did not observe any reduction or delay in NL apoptosis in hypoxic murine BMDNLs relative to normoxic BMDNLs. It has been suggested before that murine BMDNLs display different AV/PI staining properties and that might explain the different results (Walmsley et al, 2005). Also, the source of PMN might explain the different result as well. NLs isolated from peripheral blood PMN might be undergoing apoptosis already by the time of their isolation and might contribute to the difference in results.

No differences between WT and *Jbo/+* BMDM in the expression of genes from panel of selected genes was observed under normoxic conditions. This panel of genes included genes for inflammatory cytokines, Evi-1 responsive genes, TGF-β responsive genes, hypoxia responsive genes as well as genes encoding key component of TGF-β and HIF pathway. This is not unexpected as it is possible that the mutation exerts its effect in

inflammatory micro-environment that would be present in the *Jbo/+* middle ear. All this data taken collectively suggests that OM in *Jbo/+* is not due to specific global immune defect or global systemic cell recruitment defects. OM seen in *Jbo/+* mice may be a result of dysregulated response of macrophages to specific inflammatory hypoxia in the *Jbo/+* middle ear which may exacerbate inflammation.

Chapter 4

5. Studies of cellular processes in *Junbo* mice

4.1- Introduction

Unstimulated normoxic BMDM from *Junbo* mice did not exhibit anomalous expression of specific Evi-1 responsive or pro-inflammatory genes or in genes involved in the HIF pathway or TGF-β pathway (Chapter 3). Hence, the complexity of the BMDM model was increased to mimic the prevalent micro-environment of the inflamed *Jbo/+* middle ear by subjecting *Junbo* BMDM to hypoxia, LPS activation and oxysterol treatment. Differential expression of a panel of hypoxia-responsive and pro-inflammatory genes in *Jbo/+* and WT BMDM was then investigated using RT-qPCR. This approach was adopted to elucidate the contribution of *Evi-1*^{A2288T} mutation to the chronic middle ear inflammation in *Jbo/+* mice and also to provide further understanding of the role of Evi-1 in the HIF pathway and the innate immune system.

4.2- Hypoxia studies: Dysregulated HIF signalling and dysregulated expression of hypoxia responsive genes *Vegf, Glut-1, Pai-1* and *Ccl2* in *Jbo/+* BMDM

As described in detail in Chapter 1, hypoxia leads to stabilization of the Hif-1α subunit of the HIF complex and activation of the HIF pathway (Lando et al, 2002; Masson et al, 2001; Rius et al, 2008). The HIF pathway modulates more than 100 hypoxia-responsive genes, which are involved in cell mediated inflammation and affect a broad range of myeloid cell functions, such as phagocytosis, myeloid cell aggregation, cytokine secretion, cell adhesion and migration (Cramer & Johnson, 2003; Lewis et al, 2000; Semenza & Wang, 1992; Vengellur et al, 2003). Hif-1α promotes NF-κB transcriptional activity through upregulation of the NF-κB activity factor (Chandel et al, 2000; Elbarghati et al, 2008). HIF-1α is also regulated (O_2 independently) at the transcriptional level through its interaction with NF-κB (Rius et al, 2008; Taylor, 2009; Walmsley et al, 2005). Furthermore, the HIF pathway interacts with the AP-1 pathway (Laderoute, 2005; Taylor, 2009; Yu et al, 2009). Both NF-κB and AP-1 are transcription factors that induce expression of a number of pro-inflammatory genes. Many key genes involved in macrophage and NLs recruitment, innate immunity and angiogenesis such as *TNF-α, CXCL8, IL-1β, NOS-2, VEGF* are upregulated in hypoxic conditions (Rius et al, 2008). Hypoxia is also known to enhance the ability of macrophages to phagocytose bacteria and reduce bacterial infection (Anand et al, 2007; Peyssonnaux et al, 2005; Zinkernagel et al, 2008). These reports show that HIF responses are adaptive in nature and are an integral part of the complex innate immune system. These reports are evidence that pathologic hypoxia found in inflammatory lesions creates a

unique micro-environment, that modulates recruitment and functioning of both macrophages and NLs. Dysregulated HIF signalling is associated with an aggressive HIF response that drives inflammation, decreases susceptibility to infection and is commonly linked with chronic diseases such as rheumatoid arthritis and fibrosis (Dehne & Brune, 2009; Zinkernagel et al, 2008).

Hypoxia has been identified as a characteristic feature of chronic OM in the *Jbo/+* and *Jf/+* mutants (Cheeseman et al, 2011). Prevalent hypoxic conditions and Hif-1α stabilization in the *Jbo/+* mucosa and ear exudates can be attributed to the high influx of NLs to the middle ear. However, generation of *Nos-2* and pro-inflammatory cytokines such as TNF-α, IL-1β may further modulate HIF signalling and promote a hypoxic micro-environment (Dehne & Brune, 2009; Frede et al, 2006).

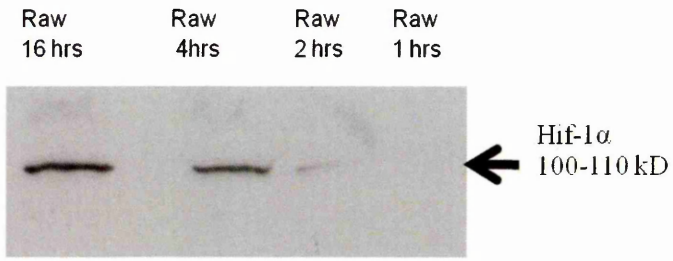
Based on these reports in combination with evidence that Evi-1 is linked to HIF signalling by Smad3 and AP-1 pathway activation (Kurokawa et al, 1998b; Tanaka et al, 1994), it was hypothesized that the *Evi-1* mutation in *Jbo/+* mice perpetuates inflammation through dysregulation of the HIF pathway. To test this hypothesis, BMDM were subjected to hypoxic conditions and relative gene expression of a panel of Evi-responsive, hypoxia-responsive, TGF-β responsive and inflammatory genes was analysed over time using RT-qPCR.

4.2.1- Hif-1α stabilization in hypoxia

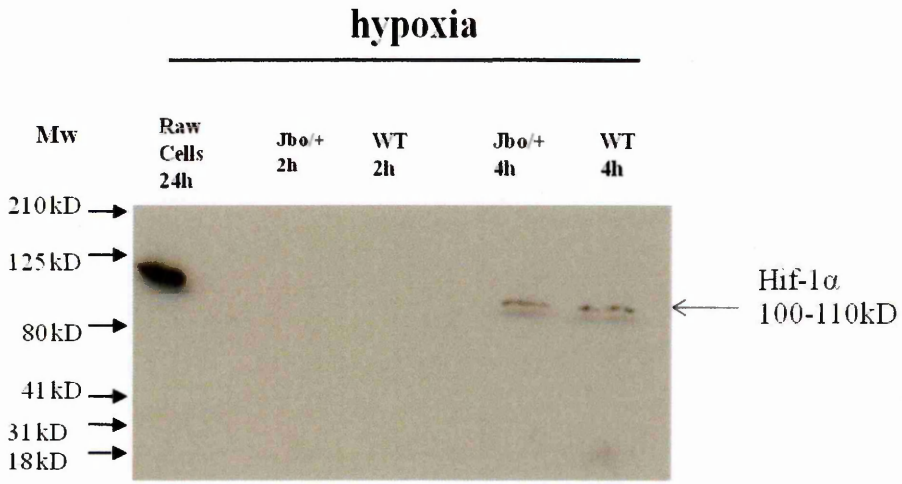
The RAW 264.7 cell line, which is derived from immortalized murine macrophages were used as an appropriate control for experimental investigation of the effects of hypoxia in BMDM. Hif-1α stabilization was investigated in RAW cells subjected to hypoxia over a time-course ranging

from 1 h to 16 h. Hif-1α expression was detected in nuclear lysates by Western blot analysis. Hif-1α protein was visualized as a band at approximately 100-110 kD in samples subjected to hypoxia for 2 h, 4 h and 16 h, demonstrating that Hif-1α protein is stabilized to detectable levels in RAW 264.7 cells from 2 h hypoxia onwards (Figure 4.1 a).

An experiment was then performed to establish the earliest optimum conditions for detection of Hif-1α stabilization and Hif-1α responsive gene expression in *Junbo* BMDM in hypoxia. BMDM were subjected to hypoxia for 2 h and 4 h and Hif-1α expression was detected in nuclear lysates by Western blot analysis. Stabilized Hif-1α was detected from 4 h in hypoxia treated *Junbo* BMDM (Figure 4.1b). RAW cells subjected to hypoxia for 24 h were used as a positive control in these experiments. A doublet band was observed in hypoxia treated BMDM. A doublet band with hypoxia treatment has been reported previously and occurs due to post translational modifications with a signal observed for unprocessed Hif-1α ~95kD and a processed Hif-1α ~116kD (Duan et al, 2005; Wang & Semenza, 1993).



a)



b)

Figure 5.1- Western blot of stabilized Hif-1α expression in Raw 264.7 and BMDM nuclear lysates under hypoxic conditions

a) Time-course of Hif-1α response to hypoxia in RAW cells. Nuclear lysates (35 μg) from hypoxia treated RAW cells were loaded in individual lanes. High Hif-1α expression was observed at 4 h and 16 h. Weak Hif-1α expression was detected at 2 h. Hif-1α expression was not detected at 1 h.

b) Nuclear lysate (35 μg) from WT and Jbo/+ BMDM subjected to hypoxia for 2 h and 4 h were loaded in individual lanes. Nuclear lysate from RAW cells subjected to hypoxia for 24 h served as a positive control. Hif-1α expression was detected at 4 h in hypoxia treated *Junbo* BMDM but not with 2 h hypoxia treatment.

4.2.2- HIF signalling is dysregulated in *Jbo/+* BMDM - Relative gene expression studies in WT and *Jbo/+* BMDM

Relative gene expression was analysed by RT-qPCR over a hypoxia time-course in *Jbo/+* and WT BMDM to determine the effect of mutation on a key panel of genes (Evi-1 responsive, hypoxia-responsive genes and inflammatory response genes) (Table 4.1). An alteration in Evi-1 responsiveness in *Jbo/+* mice might indicate direct effect of the *Evi-1*^{A2288T} mutation on the transcriptional activity of Evi-1. An anomaly in the expression of hypoxia responsive and pro-inflammatory genes would indicate that the *Evi-1*^{A2288T} mutation in *Jbo/+* BMDM does dysregulate HIF signalling under hypoxic conditions. Each experiment was replicated on three separate occasions. Each replicate represented BMDM differentiated for 7-9 d from pooled BM isolated from five individual mice (aged 6-8 wk). Although Hif-1α stabilization in response to hypoxia was detected in BMDM from *Junbo* mice from 4 h, later time-points (24, 48 and 72 h) were analysed to mimic the prolonged hypoxic conditions present in the *Jbo/+* middle ear during prolonged periods of inflammation. Relative gene expression was calculated using delta C_T (ΔC_T) and expressed as RQ or fold-change compared with respective genotypic control calibrators. Prolonged time course of 24 h, 48 h and 72 h was studied till a seven to nine day endpoint. RQ is henceforth always calibrated within the genotype to the untreated normoxic control.

Table 5.1- Panel of genes for studying cellular processes in *Jbo/+* BMDM showing cellular function of each gene

Gene	Full name	Function
Ccl2	*chemokine C-C motif ligand 2*	Chemokine
Cxcl2	*chemokine C-X-C ligand 2*	Chemokine
Evi-1	*Ecotropic viralintegration site-1*	Transcription factor
Fos	*FBJ murine osteosarcoma homolog*	Transcription factor, apoptosis, survival, proliferation. Transformation
Gata-2	*Gata binding protein 2*	Transcription factor, development, proliferation of hematopoietic cells
Glut-1	*Glucose transporter-1*	Survival
Hif-1α	*Hypoxia inducible factor-1alpha*	Transcription factor
Il-1β	*Interleukin-1beta*	Proinflammatory
Il-6	*Interleukin 6*	Proinflammatory
Abca1	*ATP binding cassetter, sub familyA member1*	
Jun	*Jun*	Transcription factor, apoptosis, survival, proliferation
Map3k14	*Mitogen activated protein kinase kinase kinase*	Kinase, participates in NF-κβ signalling
Nos-2	*Nitric oxide synthase 2, inducible*	Proinflammatory
Pai-1	*Plasminogen activator inhibitor type 1*	Proteinase inhibitor of plasminogen activator
Phd2	*Prolyl hydroxylase domain-containing protein 2*	Hif-1alpha degradation in normoxia
Smad3	*Smad3*	Transcription factor
Tnf-α	*Tumour necrosis factor-alpha*	Proinflammatory
Vegf	*Vascular endothelial growth factor*	Angiogenesis

4.2.3- Effect of *Evi-1*^{A2288T} mutation on Evi-1 responsive genes in hypoxia

Gene expression of *Evi-1* and Evi-1 responsive genes (*Gata-2, Map3k14, c-Jun* and *Fos*) were analysed using RT-qPCR. Detection of *Evi-1* gene expression required pre-amplification PCR as the levels were below the level of sensitivity for detection by traditional RT-qPCR. *Ppia* was used as an endogenous control.

Evi-1 expression (Figure 4.2) was downregulated to 0.3 fold in hypoxia treated BMDM at 72 h. No changes (>2 fold up or down) in *Evi-1* expression were observed prior to this point. No differences were observed between the *Jbo/+* and WT BMDM.

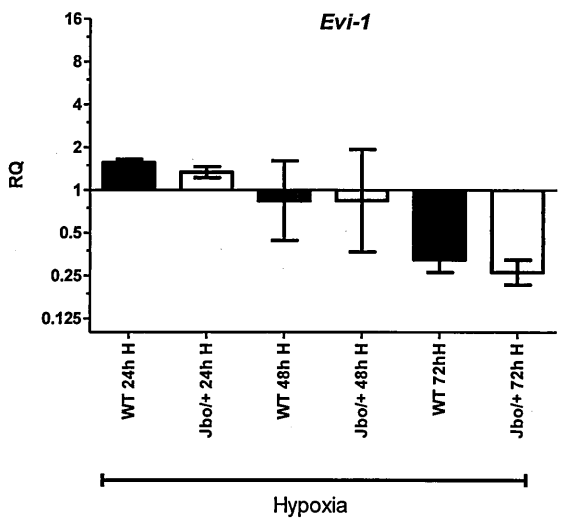


Figure 5.2-Comparable *Evi-1* expression levels in WT and *Jbo/+* BMDM after treatment with hypoxia

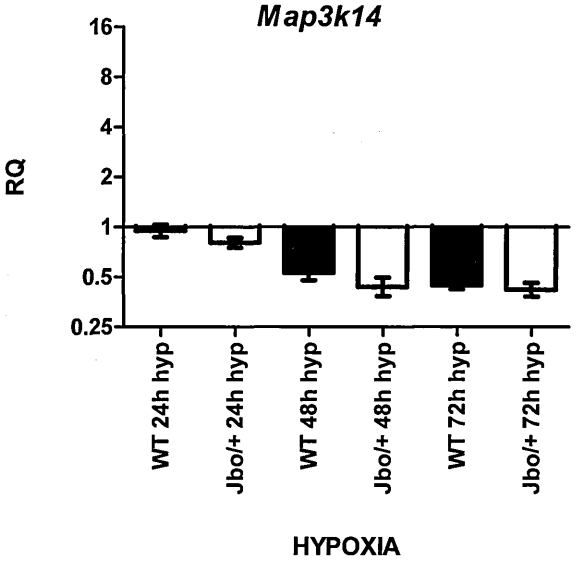
This figure illustrates relative *Evi-1* gene expression levels between WT and *Jbo/+* BMDM treated with hypoxia at 24 h, 48 h and 72 h. Blue bars denote WT levels and red bars denote *Jbo/+* levels. The error bars represent RQ min and RQ max. Gene expression data obtained was normalized to *Ppia* gene expression, n= 3 where each replicate was from a pool of 5-6 mice.

Map3k14 and *Gata-2* gene expression (Figures 4.3a and 4.3b) was time-dependently downregulated with hypoxia in both genotypes with maximum downregulation observed at 72 h hypoxia with a 0.4 fold expression in both genotypes. No differences were observed between the *Jbo/+* and WT BMDM. These data indicated that that *Map3k14* and *Gata-2* are hypoxia responsive genes which are downregulated on exposure to prolonged hypoxia. This cannot be attributed to cells dying as the RNA yield was unaltered.

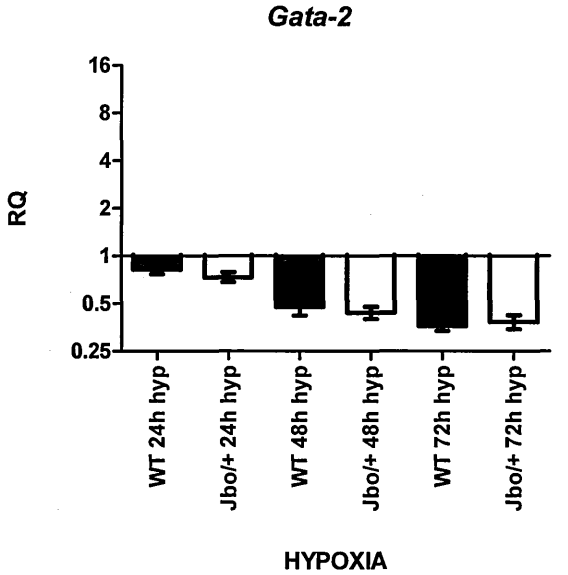
Fos gene expression (Figure 4.3c) was upregulated with 24 h hypoxia (2 fold in WT vs. 1.8 fold in *Jbo/+* BMDM) although expression returned to basal level at 48 h. No differences were observed between the *Jbo/+* and WT BMDM. Interestingly, upregulation of *Fos* gene expression was observed following further prolongation of hypoxic treatment at 72 h in WT but not in *Jbo/+* BMDM (1.5 fold in WT vs. 1.2 fold in *Jbo/+* BMDM). Although this difference was significant ($P < 0.05$) the upregulation observed in WT BMDM at 72 h falls short of the generally accepted 2-fold threshold true difference (calculation described in chapter 2), thus casting doubt on the biological relevance of this observation.

Expression of another Evi-1 responsive gene, *Jun,* (Figure 4.3d) was upregulated in response to hypoxia with a peak expression observed at 48 h of hypoxia (2.2 fold in WT vs. 2.0 fold in *Jbo/+*). No differences in *Jun* expression were observed between the *Jbo/+* and WT BMDM at 24 h and 48 h. However, a significant difference in *Jun* induction was observed at 72 h hypoxia (1.9 in WT vs. 1.6 in *Jbo/+* BMDM; $P < 0.05$). However the change was small and the induction of *Jun*, falls short of 2-fold threshold, indicating that the biological relevance of this difference is questionable.

a)


Map3k14
RQ
16
8
4
2
1
0.5
0.25
WT 24h hyp
Jbo/+ 24h hyp
WT 48h hyp
Jbo/+ 48h hyp
WT 72h hyp
Jbo/+ 72h hyp
HYPOXIA

b)


Gata-2
RQ
16
8
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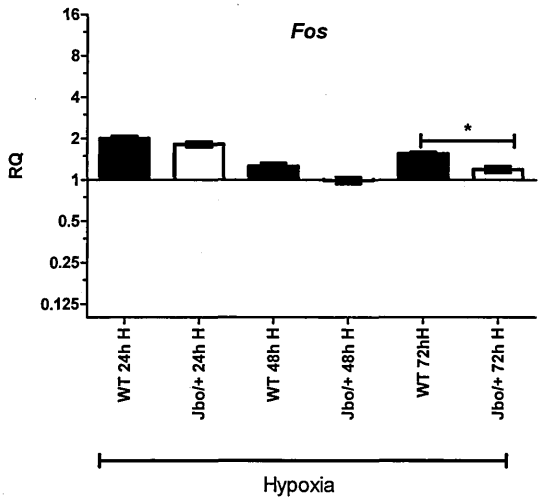
WT 24h hyp
Jbo/+ 24h hyp
WT 48h hyp
Jbo/+ 48h hyp
WT 72h hyp
Jbo/+ 72h hyp
HYPOXIA

c)


Fos
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WT 24h H
Jbo/+ 24h H
WT 48h H
Jbo/+ 48h H
WT 72hH
Jbo/+ 72h H
Hypoxia

d)

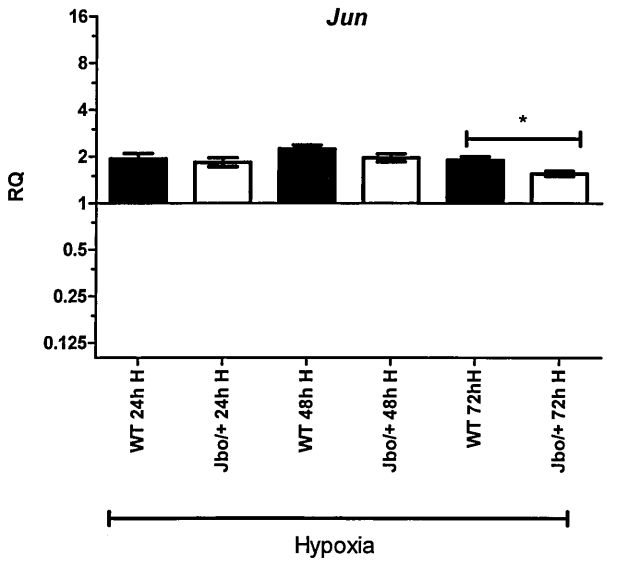


Figure 5.3- Relative expression of Evi-1 responsive genes in hypoxia treated WT and *Jbo/+* BMDM

This figure illustrates relative gene expression levels between WT and *Jbo/+* BMDM treated with hypoxia at 24 h, 48 h and 72 h. Figure panels show relative (a) *Map3k14* gene expression, (b) *Gata-2* gene expression (c) *Fos* gene expression and (d) *Jun* gene expression between the two genotypes, n=3. Each replicate was obtained from 8 d old BMDM from a pool of 5-6 individual mice (6-8 wk old). Statistics were performed using RQ min-max as recommended by Applied Biosystems using the ABI 7500 sds software. The bars represents fold increase (RQ) in gene expression observed in hypoxia treated BMDM over the normoxic control calibrator of respective genotype. Blue bars denote WT levels and red bars denote *Jbo/+* levels. The error bars represent RQ min and RQ max. Gene expression data obtained was normalized to *Ppia* gene expression. * $P < 0.05$.

4.2.4- The A2288T *Evi-1* mutation in *Jbo/+* induces altered expression of *Vegf, Glut-1* and *Pai-1* in hypoxia

Relative gene expression levels of *Hif-1α* and hypoxia and TGF-β responsive genes (*Phd2*, *Vegf*, *Glut-1*, and *Pai*-1) were compared in WT and *Jbo/+* BMDM using RT-qPCR. According to my hypothesis that HIF signalling in *Jbo/+* mice results in aberrant HIF and TGF-β signalling; hypoxia responsive genes and TGF-β responsive genes were chosen for this study.

Hypoxia did not have any impact on *Hif-1α* gene expression in WT and *Jbo/+* BMDM (Figure 4.4).

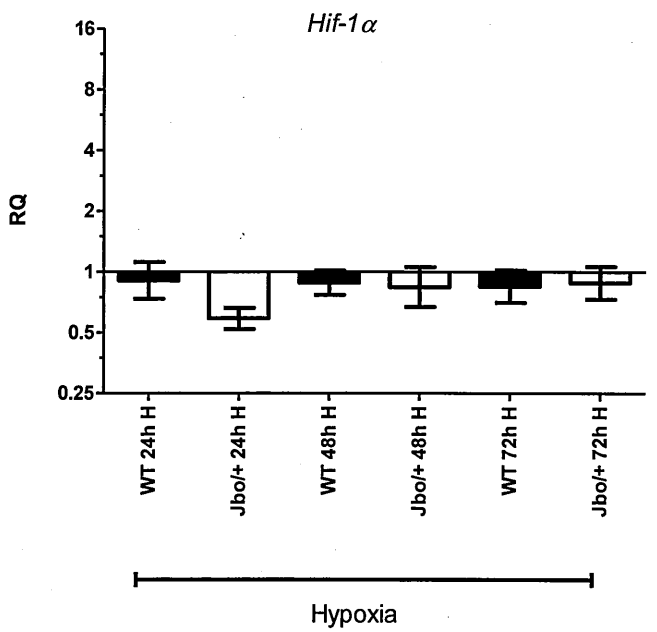


Figure 5.4- Comparable *Hif-1α* expression levels in WT and *Jbo/+* BMDM after treatment with hypoxia

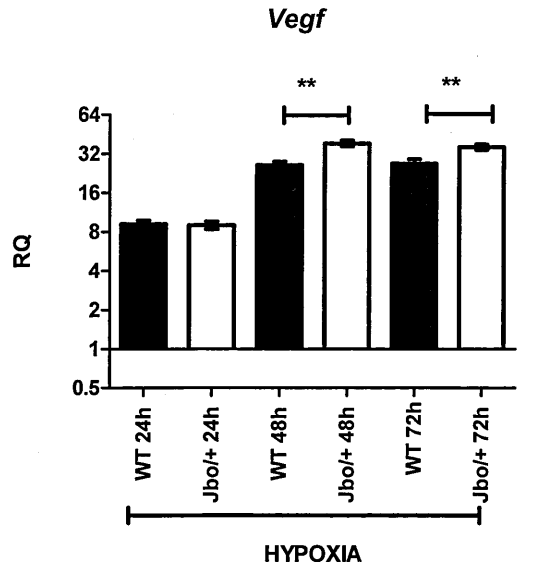
This figure illustrates relative *Hif-1α* gene expression levels between WT and *Jbo/+* BMDM treated with hypoxia at 24 h, 48 h and 72 h. Blue bars denote WT levels and red bars denote *Jbo/+* levels. The error bars represent RQ min and RQ max. Gene expression data obtained was normalized to *Ppia* gene expression. Three biological replicates were used for the study where each replicate was from a pool of 5-6 mice.

Vegf gene is involved in angiogenesis and its expression increased in response to hypoxia in a time-dependent manner (Figure 4.5a). *Vegf* expression was increased in both genotypes at 24 h (9.2 in WT vs. 9.0 in *Jbo*/+ BMDM). *Vegf* levels were further increased on prolonged hypoxia exposure of 48 h (26.2 vs. 38.4 *Jbo*/+ BMDM) and 72 h (26.8 in WT vs. 36.0 in *Jbo*/+ BMDM) with a significant difference ($P < 0.01$) observed in *Vegf* expression levels between genotypes.

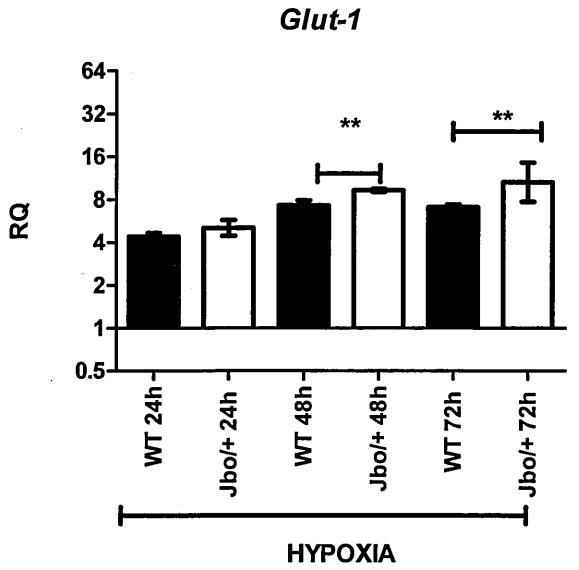
Glut-1 gene expression also increased in response to hypoxia in a time-dependent manner (Figure 4.5b). *Glut-1* expression was increased at 24 h (4.4 in WT vs. 5.1 in *Jbo*/+ BMDM) with no significant difference between the genotypes. *Glut-1* levels were further increased on prolonged hypoxia exposure at 48 h (7.3 fold in WT vs. 9.3 in *Jbo*/+ BMDM) and 72 h (7.1 fold in WT and 10.6 fold in *Jbo*/+ BMDM; $P <0.01$).

Upregulation of *Phd2* was also observed in response to hypoxia in a time-dependent manner (Figure 4.5c) with the expression levels increasing from 24 h hypoxia (5.0 fold in WT vs. 4.7 fold in *Jbo*/+ BMDM) to prolonged hypoxia exposure at 72 h (7.0 fold WT vs. 6.6 fold *Jbo*/+ BMDM). No significant differences in *Phd2* expression were observed between the two genotypes at any point.

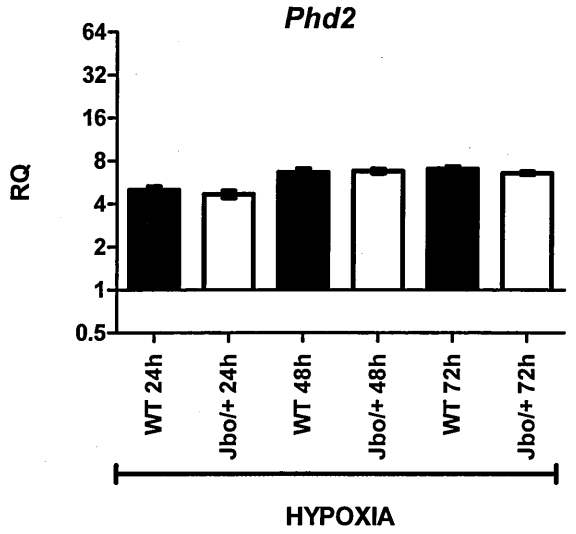
Pai-1 expression was induced after a relatively short hypoxia exposure of 24 h. A significant difference between the genotypes was observed at this point (6.9 fold WT vs. 5.4 fold *Jbo*/+; $P<0.01$) (Figure 4.5d). *Pai-1* expression declined with longer hypoxia exposure to the point that no differences were detectable between the genotypes at 48 h (Figure 4.5d). However, a significant differences in downregluated *Pai-1* level was detected at 72 h hypoxia (0.5 vs. 0.7 *Jbo*/+ BMDM; $P <0.01$).


Vegf
**
**
64
32
16
8
4
2
1
0.5
RQ
WT 24h
Jbo/+ 24h
WT 48h
Jbo/+ 48h
WT 72h
Jbo/+ 72h
HYPOXIA

a)


Glut-1
**
**
64
32
16
8
4
2
1
0.5
RQ
WT 24h
Jbo/+ 24h
WT 48h
Jbo/+ 48h
WT 72h
Jbo/+ 72h
HYPOXIA

b)


Phd2
64
32
16
8
4
2
1
0.5
RQ
WT 24h
Jbo/+ 24h
WT 48h
Jbo/+ 48h
WT 72h
Jbo/+ 72h
HYPOXIA

c)

d)

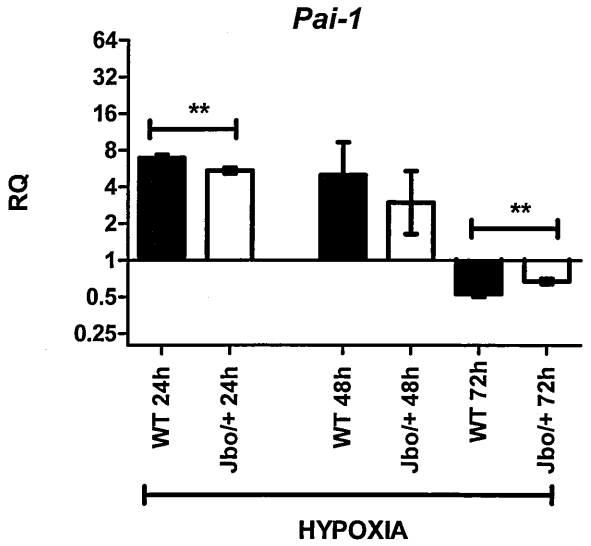


Figure 5.5- Relative expression of hypoxia-responsive genes in hypoxia treated WT and *Jbo/+* BMDM

This figure illustrates relative gene expression levels between WT and *Jbo/+* BMDM treated with hypoxia at 24 h, 48 h and 72 h. Three biological replicates were used for the study where each replicate was from a pool of 5-6 mice. Figure panels show relative (a) *Vegf* gene expression, (b) *Glut-1* gene expression (c) *Phd2* gene expression and (d) *Pai-1* gene expression between the two genotypes. Gene expression data obtained was normalized to *Ppia* gene expression. ** $P < 0.01$.

4.2.5- Inflammatory response gene panel

It was hypothesized that differential gene expression of the key inflammation markers, *Il-6*, *Il-1β* and *Tnf-α* in hypoxia treated *Jbo/+* BMDM would be indicative of a dysregulated inflammatory response to hypoxia, indicating perpetuation of inflammation.

Tnf-α expression (Figure 4.6a) was not affected by hypoxia treatment at 24 h although expression was downregulated in both genotypes at 48 h (0.5 fold in both genotypes) and 72 h (0.6 WT vs. 0.7 in *Jbo/+*) respectively. No significant differences were observed between the genotypes.

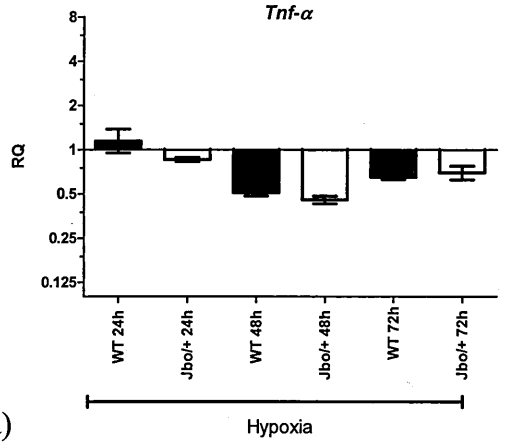
Il-1β expression (Figure 4.6b) increased following 24 h hypoxia treatment in both genotypes (2.0 fold WT vs. 1.8 fold in *Jbo/+* BMDM). Expression gradually decreased to approximate basal levels with further exposure. A significant difference ($P < 0.05$) in *Il1-β* expression was observed at 48 h (1.2 fold in WT vs. 0.6 fold in *Jbo/+* BMDM).

Il-6 expression (Figure 4.6c) was not affected by hypoxia until treatment was prolonged to 72 h where upregulation was observed in both WT and *Jbo/+* BMDM (1.6 fold WT vs. 1.8 fold in *Jbo/+* BMDM). No significant differences were observed between the genotypes.

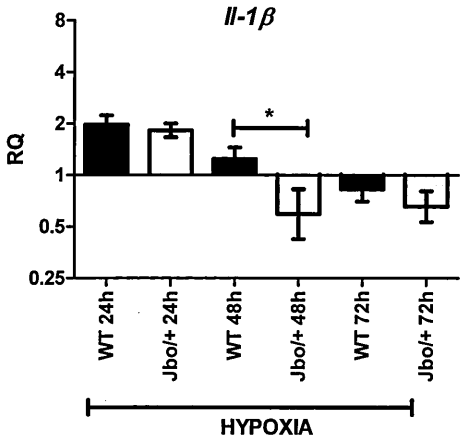
Nos-2 expression (Figure 4.6d) was increased in BMDM under hypoxic conditions from 24 h (3.9 WT vs. 3.5 *Jbo/+* BMDM; $P < 0.05$) to 48 h (2.8 fold in WT vs. 3.2 fold in *Jbo/+* BMDM). A significant difference between Nos-2 levels was also observed at 72 h hypoxia (1.2 fold in WT vs. 1.3 fold in *Jbo/+* BMDM; $P < 0.05$). However the differences between the genotypes were small and less than 25% and beneath the 2-fold cut off threshold.

No changes in *Cxcl2* expression (Figure 4.6e) were observed in response to hypoxia in *Jbo/+* BMDM and WT BMDM. Although a modest increase (1.5 fold in WT vs. 1.4 fold in *Jbo/+*) in *Cxcl2* expression was observed in both genotypes at 48 h. A significant difference (1.1 fold WT vs. 0.9 fold *Jbo/+*; $P<0.05$) was observed in Cxcl2 expression at 72 h but the difference was very minute to be biologically relevant.

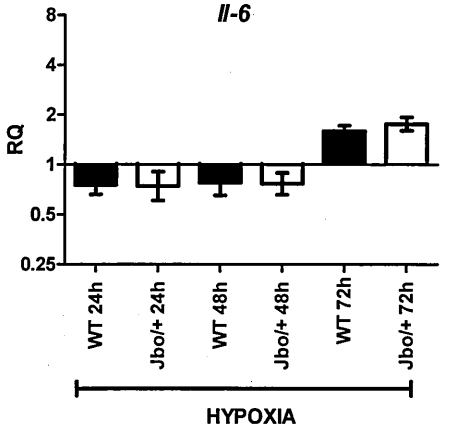
Ccl2 gene expression decreased in response to hypoxia in a time-dependent manner (Figure 4.6f). *Ccl2* expression levels between WT and *Jbo/+* BMDM at 24 h hypoxia (0.7 fold WT vs. 0.5 fold *Jbo/+* BMDM; $P < 0.05$) as levels in *Jbo/+* BMDM and 72 h hypoxia (0.2 fold WT vs. 0.3 fold *Jbo/+* BMDM; $P < 0.05$) were significantly different. However, no significant difference was observed at 48 h hypoxia.


Tnf-α
RQ
8
4
2
1
0.5
0.25
0.125
WT 24h
Jbo/+ 24h
WT 48h
Jbo/+ 48h
WT 72h
Jbo/+ 72h
Hypoxia

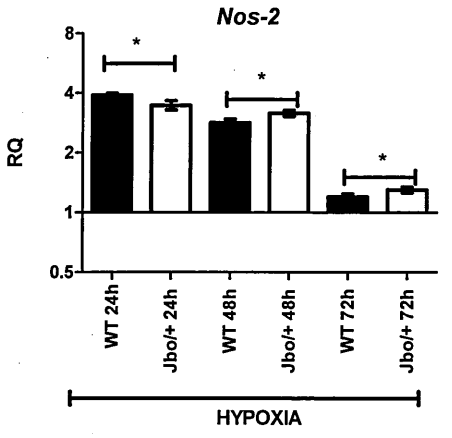
a)


Il-1β
RQ
8
4
2
1
0.5
0.25
*
WT 24h
Jbo/+ 24h
WT 48h
Jbo/+ 48h
WT 72h
Jbo/+ 72h
HYPOXIA

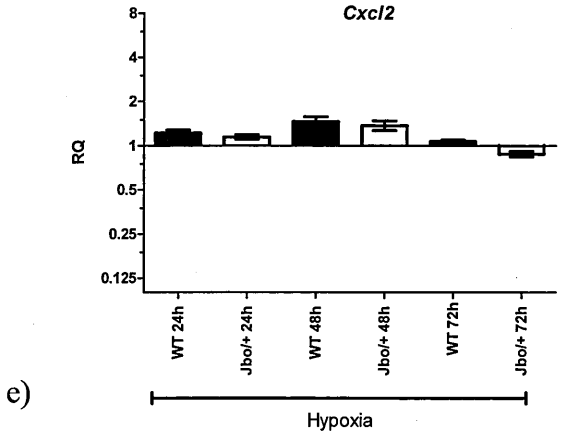
b)


Il-6
RQ
8
4
2
1
0.5
0.25
WT 24h
Jbo/+ 24h
WT 48h
Jbo/+ 48h
WT 72h
Jbo/+ 72h
HYPOXIA

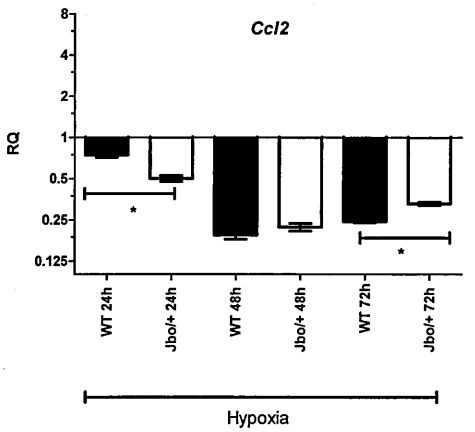
c)


Nos-2
RQ
8
4
2
1
0.5
*
*
*
WT 24h
Jbo/+ 24h
WT 48h
Jbo/+ 48h
WT 72h
Jbo/+ 72h
HYPOXIA

d)



e)



f)

Figure 5.6- Relative expression of inflammatory genes in hypoxia treated WT and *Jbo/+* BMDM

This figure illustrates relative gene expression levels of pro-inflammatory genes between WT and *Jbo/+* BMDM treated with hypoxia at 24 h, 48 h and 72 h. Three biological replicates were used for the study. Each replicate was obtained from 8d old BMDM from a pool of 5-6 individual mice (6-8 wk old). Figure panels show relative (a) *Tnf-α* gene expression, (b) *Il-1β* gene expression (c) *Il-6* gene expression (d) *Nos-2* gene expression, (e) *Ccl2* gene expression and (f) *Cxcl2* gene expression between the two genotypes. Gene expression data obtained was normalized to *Ppia* gene expression. * $P < 0.05$.

Table 4.2- Effect of hypoxia on WT and *Jbo*/+ BMDM

Gene	Hypoxia					
	WT 24h	*Jbo*/+ 24h	WT 48 h	*Jbo*/+ 48 h	WT 72 h	*Jbo*/+ 72 h
Ccl2	0.7 (0.7, 0.8)	0.5 (0.5, 0.5)	0.2 (0.2, 0.2)	0.2 (0.2, 0.2)	0.2 (0.2, 0.2)	0.3 (0.3, 0.3)
Cxcl2	1.2 (1.2, 1.3)	1.1 (1.1, 1.2)	1.5 (1.4, 1.6)	1.4 (1.3, 1.5)	1.1 (1.0, 1.1)	0.9 (0.8, 0.9)
Evi-1	1.6 (1.5, 1.6)	1.3 (1.2, 1.5)	0.8 (0.4, 1.6)	0.8 (0.4, 1.9)	0.3 (0.3, 0.4)	0.3 (0.2, 0.3)
Fos	2.0 (1.9, 2.1)	1.8 (1.7, 1.9)	1.3 (1.2, 1.3)	1.0 (0.9, 1.0)	1.5 (1.5, 1.6)	1.2 (1.1, 1.3)
Gata-2	0.8 (0.8, 0.9)	0.7 (0.7, 0.8)	0.5 (0.4, 0.5)	0.4 (0.4, 0.5)	0.4 (0.3, 0.4)	0.4 (0.3, 0.4)
Glut-1	4.4 (4.1, 4.6)	5.1 (4.5, 5.7)	7.3 (6.7, 7.9)	9.3 (9.1, 9.5)	7.1 (6.8, 7.4)	10.6 (7.7, 14.6)
Hif-1α	0.9 (0.7, 1.1)	0.6 (0.5, 0.7)	0.9 (0.8, 1.0)	0.8 (0.7, 1.1)	0.8 (0.7, 1.0)	0.9 (0.7, 1.1)
Il-1β	2.0 (1.7, 2.2)	1.8 (1.7, 2.0)	1.2 (1.1, 1.5)	0.6 (0.4, 0.8)	0.8 (0.7, 1.0)	0.7 (0.5, 0.8)
Il-6	0.8 (0.7, 0.9)	0.7 (0.6, 0.9)	0.8 (0.7, 0.9)	0.8 (0.7, 0.9)	1.6 (1.5, 1.7)	1.8 (1.6, 1.9)
Jun	1.9 (1.8, 2.1)	1.8 (1.7, 2.0)	2.2 (2.1, 2.4)	2.0 (1.8, 2.1)	1.9 (1.8, 2.0)	1.6 (1.5, 1.6)
Map3k14	0.9 (0.9, 1.0)	0.8 (0.8, 0.9)	0.5, 0.5, 0.6)	0.4 (0.4, 0.5)	0.4 (0.4, 0.5)	0.4 (0.4, 0.5)
Nos-2	3.9 (3.8, 4.0)	3.5 (3.3, 3.6)	2.8 (2.7, 3.0)	3.2 (3.1, 3.3)	1.2 (1.2, 1.2)	1.3 (1.3, 1.3)
Pai-1	6.9 (6.6, 7.4)	5.4 (5.1, 5.7)	5.0 (2.7, 9.3)	3.0 (1.6, 5.4)	0.5 (0.5, 0.6)	0.7 (0.6, 0.7)
Phd2	5.0 (4.7, 5.3)	4.7 (4.4, 4.9)	6.6 (6.2, 7.1)	6.8 (6.5, 7.0)	7.0 (6.7, 7.3)	6.6 (6.4, 6.7)
Tnf-α	1.1 (0.9, 1.4)	0.9 (0.8, 0.9)	0.5 (0.5, 0.5)	0.5 (0.4, 0.5)	0.6 (0.6, 0.7)	0.7 (0.6, 0.8)
Vegf	9.2 (8.6, 9.8)	9.0 (8.4, 9.6)	26 (24.6, 27.8)	38.4 (36.5, 40.4)	26.8 (24.7, 29.1)	36 (34.4, 37.8)

This table shows the fold change in treated WT and *Jbo*/+ BMDM observed after hypoxia treatment of *Junbo* BMDM at 24 h, 48 h and 72 h. Coloured numerical values (blue and red) represent significant difference between genotype at that time point ($P < 0.05$). Numerical figures in blue were not considered biologically relevant due to the relatively small difference between the genotypes (<25%) and did not make the cut-off threshold of 2 fold up/downregulation. Numerical values in red were considered biologically relevant and displayed a strong trend.

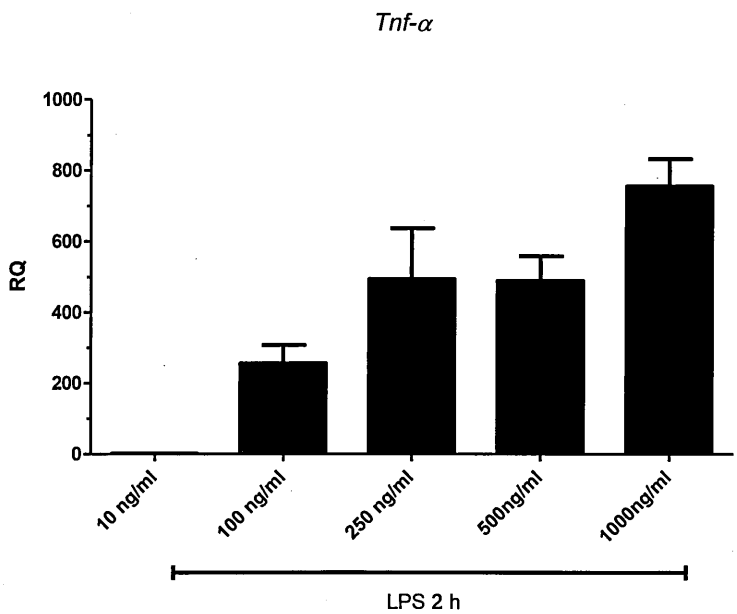
4.3- LPS studies: Activated *Jbo/+* BMDM have altered gene expression profiles for *Tnf-α, Il-1β, Vegf* and *Il-6*

Jbo/+ mice exhibit chronic inflammation in the middle ear. LPS challenge is a classic model for investigating inflammatory processes. As mentioned in Chapter 1, macrophages exist in a number of activation states that are associated with differential gene expression depending on the activating signal they are exposed to (Laskin et al, 2011). LPS, a glycolipid component of bacterial cell wall is a potent trigger for macrophage activation and results in activation of phagocytic cells such as monocytes/macrophages and neutrophils (Dobrovolskaia & Vogel, 2002). LPS recognition by receptors such as TLRs induces MyD88 dependent activation of the NF-κB pathway (Takeda & Akira, 2004). NF-κB is a transcription factor that regulates expression of *IL-1β, IL-6, IL-8, TNF-α, NOS-2* (Guha & Mackman, 2001). LPS also activates transcription factors involved in the AP-1 and MAPK signalling pathways (Guha & Mackman, 2001) resulting in release of cytokines that regulate the stages of inflammation and resolution or initiation of chronic conditions such as otitis media (Smirnova et al., 2002). LPS is also known to act as a non-hypoxic stimulus that strongly upregulates the HIF-1 complex in normoxia and modulates expression of hypoxia-responsive genes (Blouin et al, 2004). Furthermore, activation of NF-κB and production of pro-inflammatory cytokines by LPS induces HIF-1α transcription and protein (Blouin et al, 2004). Some of these pro-inflammatory genes upregulated on LPS activation of macrophages, such as *TNF-α* and *Il-1β*, further activate HIF-1 at the translational level (Brune & Zhou, 2007; Dehne & Brune, 2009; Hellwig-Burgel et al, 1999). This cross-talk with the NF-κB pathway serves to enhance Hif-1α transcriptional activity and is known to upregulate hypoxia-responsive genes such as *Vegf*

and *Glut-1,* even under normoxic conditions (Elson et al, 2000). HIF-1 is also reported to be a mediator of inflammatory responses (Cramer & Johnson, 2003; Cramer et al, 2003; Peyssonnaux et al, 2005; Walmsley et al, 2005). These observations provide crucial evidence of a link between HIF signalling and NF-κB signalling. It can be hypothesized that the *Jbo/+* mutation A2288T in *Evi-1* influences both of these major signalling pathways via Evi-1 leading to alterations in the functional role of activated macrophages. This is supported by evidence demonstrating that bacterial load decreases the age of disease onset in *Jbo/+* mice (Parkinson et al, 2006). It is hypothesized that chronic inflammation in *Jbo/+* mice is caused by a dysregulated macrophage activation profile leading to defective resolution of inflammation.

In order to better mimic the *in vivo* conditions of middle ear and to study responses to inflammation and/or hypoxia in *Jbo/+* BMDM, LPS challenged cells were exposed to hypoxia. LPS challenge on hypoxic cells was also performed to study effect of hypoxia conditioning on LPS-responsiveness of BMDM. The results from hypoxia challenge suggests a predisposition of *Jbo/+* BMDM towards a dysregulated HIF signalling and it is important to see if this dysregulation is manifested in inflammatory conditions. To determine the effects of the A2288T *Evi-1* mutation in *Junbo* mice on the functionality of activated macrophages, the following experiments were performed. Expression of Evi-1 protein was initially investigated to discount the existence of abnormal Evi-1 expression in *Jbo/+* BMDM following classical activation of *Junbo* BMDM. The LPS dosage optimisation and time-course studies using RT-qPCR and *Tnf-α* assays (10 ng/ml, 100 ng/ml, 250 ng/ml, 500 ng/ml and 1000 ng/ml) (Figure 4.7) were performed to define the optimal conditions for subsequent

experiments. BMDM were stimulated with a standard LPS dose (250 ng/ml) over 2 time points representing acute (2 h) and prolonged (24 h) exposure under defined conditions of normoxia and hypoxia. Analysis of exposure for 72 h was not included as the RNA yield was prohibitively low due to pyroapoptosis (Fink & Cookson, 2005). Gene and protein expression of key inflammatory response genes were then analysed using the ΔC_T method to provide an insight into the mechanism of chronic inflammation in middle ear in *Jbo/+* mice.



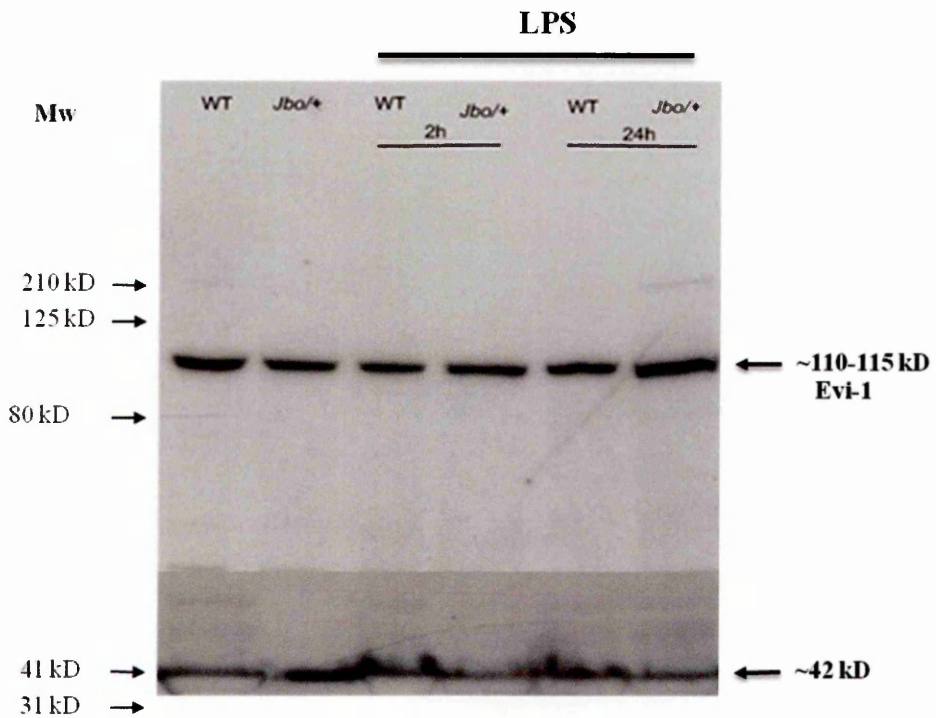
	10 ng/ml	100 ng/ml	250 ng/ml	500ng/ml	1000ng/ml
RQ	1.3	255.5	493.3	488.2	755.3
RQMin	1.2	212.4	382.2	426.7	685.5
RQMax	1.4	307.3	636.6	558.5	832.2

Figure 5.7- LPS dosage optimisation

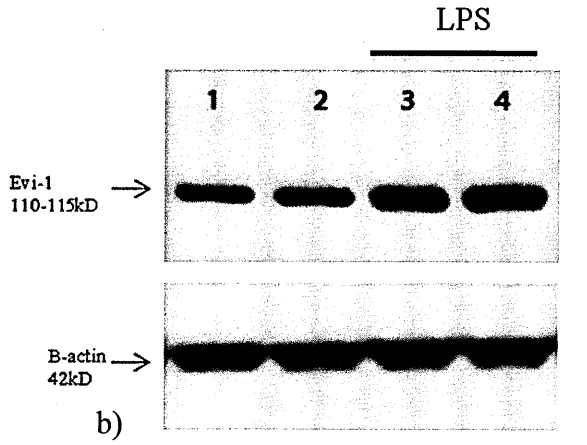
Pooled BMDM isolated from WT mice (n = 6) were used in this experiment. Bars represent fold-increase in *Tnf-α* gene expression in LPS (10 to 1000 ng/ml) treated WT and *Jbo/+* BMDM compared with control untreated, genotype-matched BMDM. The y axis is presented on a Log_2 scale and shows fold-changes (RQ) compared with genotype-matched controls. Error bars represent RQ min and RQ max, which were calculated using three technical replicates. Gene expression data obtained were normalized to *Ppia* gene expression.

4.3.1- Evi-1 expression is comparable in WT and *Jbo/+* BMDM upon classical activation

Upregulated *Evi-1* expression in response to LPS stimulation has been reported to reach a maximum after 2 h (Wells et al, 2003). *Evi-1* and Evi-1 expression are not affected in resting *Junbo* BMDM (Chapter 3). Therefore, LPS-induced activation of BMDM was investigated to assess the effects of the A2288T *Evi-1* mutation in *Jbo/+* mice. Results of these studies indicated that Evi-1 protein expression was upregulated in response to LPS treatment by 15% in both WT and *Jbo/+* BMDM compared with genotype-matched controls (Figure 5.8).



a)



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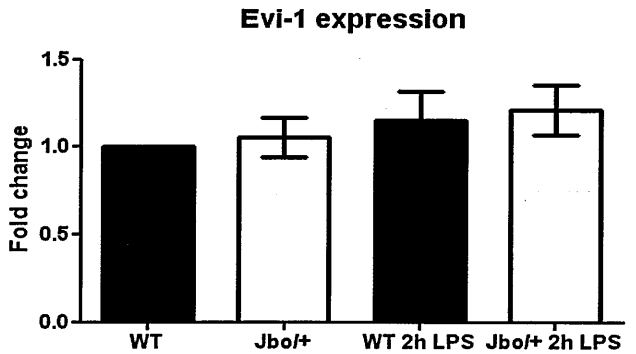


c)

Figure 5.8- Evi-1 is expressed equally in both WT and *Jbo/+* BMDM

a) Western blot analysis of Evi-1 (115 kD) expression in total cell lysates from WT and *Jbo/+* BMDM activated with and without LPS (2 h and 24 h). Protein (20 µg) was loaded in individual lanes. Protein extraction was performed with Nucleospin kits.
b) Western blot and densitometric analysis of Western blots for Evi-1 at basal level and after 2 h LPS activation. Lane 1-unstimulated WT, Lane 2-unstimulated *Jbo/+*, Lane 3- LPS treated WT, Lane 4- LPS treated *Jbo/+*. No difference in Evi-1 expression was detected between the genotypes. Results are representative of three individual western blot experiments. Error bars denote SEM. Fold-change over WT (for *Jbo/+*) and genotype-matched untreated controls was calculated by measuring mean pixel density using Vision Works LS on Chemidoc. LPS increased *Evi-1* expression by 15% in both WT and *Jbo/+* BMDM.

4.3.2- Relative gene expression studies in LPS activated *Junbo* BMDM reveals altered *Vegf, Il-6, Il-1β* and *Tnf-α* expression in *Jbo/+* BMDM

Gene and protein expression of key inflammatory response genes (*Tnf-α, Il-6, Il-1β*) and Hif-1α and hypoxia responsive genes (*Vegf, Jun, Nos-2*) were analysed in *Jbo/+* BMDM and WT BMDM after LPS treatment in normoxia and hypoxia by RT-qPCR to provide an insight into the mechanism of chronic inflammation in the middle ear in *Jbo/+* mice (Table 4.3).

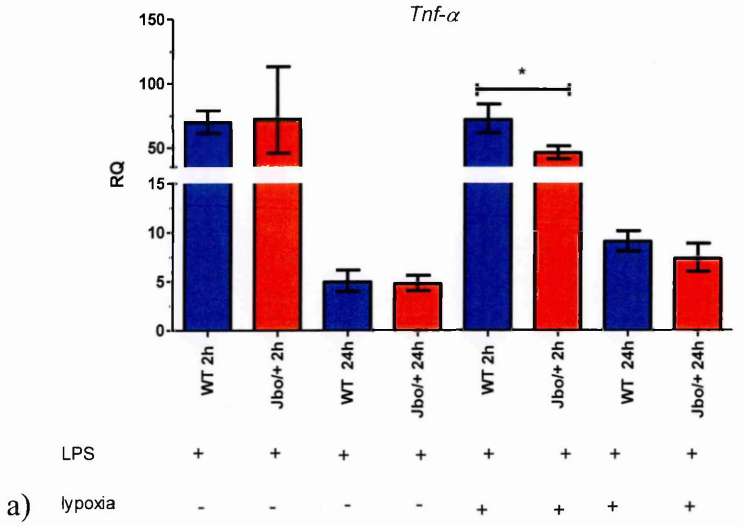
Tnf-α expression (Figure 4.9a) was upregulated at 2 h LPS treatment (69.8 fold in WT vs. 72.2 fold in *Jbo/+* BMDM) and the induction was reduced by 24 h LPS activation (4.9 fold WT vs. 4.8 fold *Jbo/+*) but there was no significant difference between the genotypes. However on LPS and hypoxia induction of *Tnf-α* expression at 2 h displayed a genotypic difference (71.6 fold WT vs. 45.8 fold *Jbo/+* BMDM; $P < 0.05$). This trend of lower *Tnf-α* levels in treated *Jbo/+* BMDM was maintained at 24 h hypoxia and LPS treatment (9.1 fold in WT vs. 7.3 fold in *Jbo/+*) but there was no significant difference between the genotypes at that time-point.

Il-6 expression (Figure 4.9b) was induced in a time-dependent manner with LPS treatment in normoxia. *Il-6* levels were comparable between the genotypes at 2 h LPS activation in normoxia (332 fold WT vs. 334 fold *Jbo/+* BMDM) but significantly different at 24 h activation (1200 fold WT vs. 1720 fold in *Jbo/+* BMDM; $P < 0.05$). The combination of hypoxia to the LPS treatment induced *Il-6* levels at 2h (1850 fold WT vs. 1547 fold in *Jbo/+* BMDM) and 24h (756 WT vs. 911 in *Jbo/+* BMDM) but no significant difference between the genotypes was observed.

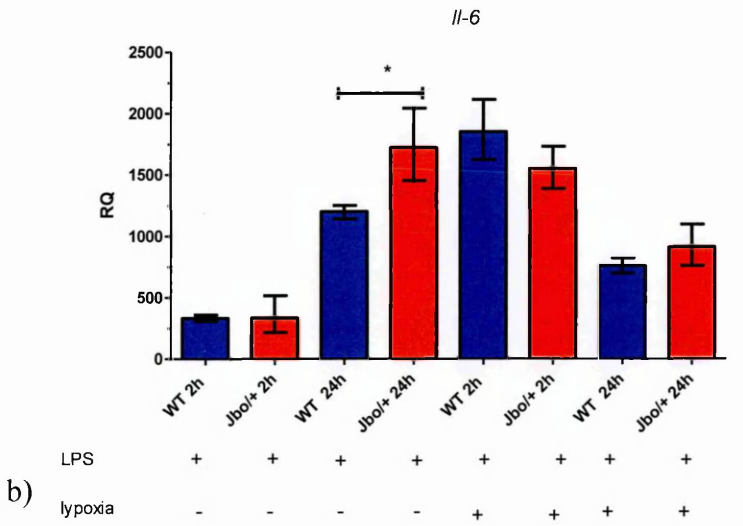
Il-1β expression (Figure 4.9c) was induced by LPS treatment in a time-dependent manner as well. Greater *Il-1β* induction was detected in response to LPS stimulation at 24 h normoxia (1049 WT vs. 1075 in *Jbo/+* BMDM)

compared with that observed at 2 h normoxia (347 WT vs. 338 in *Jbo/+* BMDM). Combination of LPS-stimulation and hypoxia resulted in reduced *Il-1β* induction in *Jbo/+* BMDM at 2 h (584 in WT vs. 415 in *Jbo/+* BMDM; $P < 0.05$). However, the difference was not significant under the 24 h hypoxia and LPS (1267 fold in WT vs. 1208 fold in *Jbo/+* BMDM).

Expression of the key hypoxia responsive gene, *Vegf*, was also analysed in LPS and hypoxia treated BMDM (Figure 4.9d). *Vegf* expression was increased by hypoxia and LPS had an additive effect on *Vegf* induction. *Vegf* expression modestly increased (1.9 WT vs. 1.7 in *Jbo/+* BMDM) in both genotypes at 2 h LPS treatment in normoxia and no significant differences were observed between the genotypes. However, *Vegf* was induced by 13.3 fold in WT and 29.1 fold in *Jbo/+* BMDM at 24 h LPS treatment in normoxia ($P < 0.05$). LPS-stimulation in hypoxia increased *Vegf* levels at 2 h (4.9 fold WT vs. 2.9 fold *Jbo/+*) and at 24 h (12.1 fold vs. 14.9 fold *Jbo/+*) but no significant genotypic difference was observed.


Tnf-α
RQ
150
100
50
15
10
5
0
*
WT 2h
Jbo/+ 2h
WT 24h
Jbo/+ 24h
WT 2h
Jbo/+ 2h
WT 24h
Jbo/+ 24h
LPS + + + + + + + +
lypoxia - - - - + + + +

a)


Il-6
RQ
2500
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1500
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500
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*
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Jbo/+ 2h
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Jbo/+ 24h
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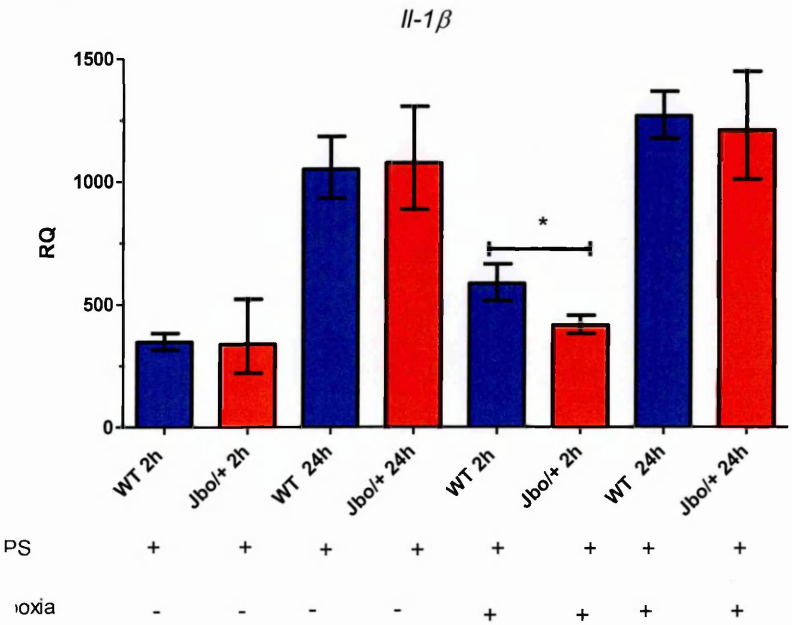
b)


Il-1β
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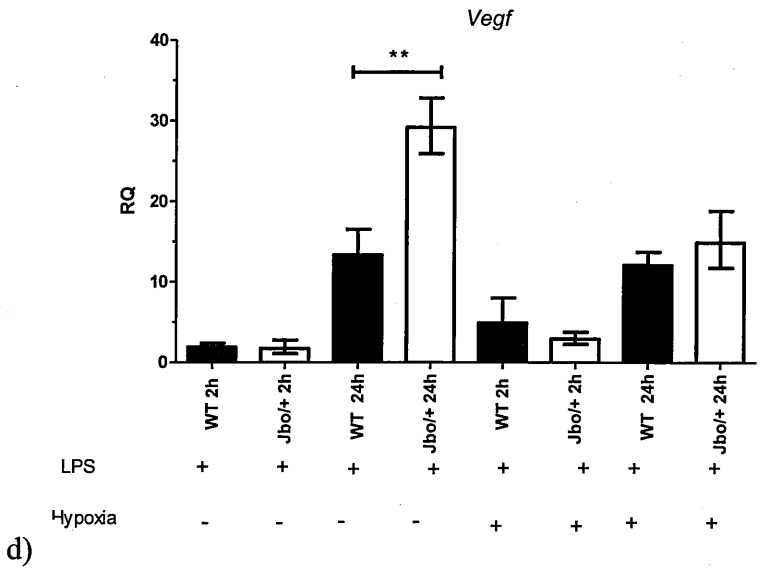
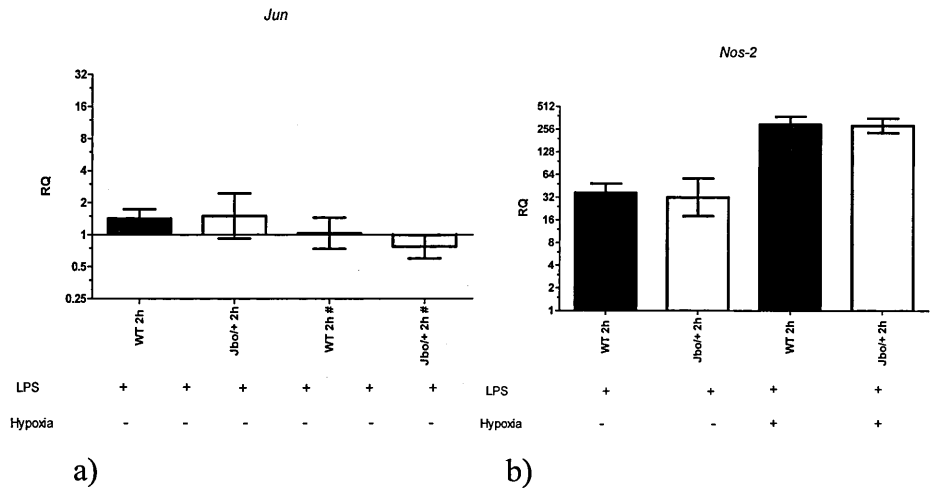


d)

Figure 5.9- Relative gene expression of *Tnf-α, Il-1ß, Il-6, Vegf* in LPS activated WT and *Jbo/+* BMDM under normoxia and hypoxia

This figure illustrates relative gene expression levels between WT and *Jbo/+* BMDM treated with 250ng/ml LPS (2 h, 24 h) in normoxia and hypoxia. Here, n=6 where each replicate was from from individual mice. Figure panels show relative (a) *Tnf-α* gene expression, (b) *Il-1ß* gene expression (c) *Il-6* gene expression and (d) *Vegf* gene expression between the two genotypes. Gene expression data obtained was normalized to *Ppia* gene expression. * $P < 0.05$, **$P < 0.01$.

Jun expression (Figure 4.10a) was not affected by LPS treatment regardless of oxygen levels at 2 h. *Nos-2* expression (Figure 4.10b) was analysed only at 2 h following LPS stimulation and was found to be increased with LPS stimulation in normoxia in both genotypes without any significant difference (37 WT vs. 32 *Jbo/+*). LPS induced *Nos-2* levels were enhanced by hypoxia at 2 h (297 fold WT vs. 287 fold *Jbo/+* BMDM) with no significant differences observed between the two both genotypes.



a) b)

Figure 5.10- Relative gene expression of *Jun* and *Nos-2* in LPS activated WT and *Jbo/+* BMDM under normoxia and hypoxia

This figure illustrates relative gene expression levels between WT and *Jbo/+* BMDM treated with 250ng/ml LPS (2 h) in normoxia and hypoxia. Here, n=6 where each replicate was from from individual mice. Figure panels show relative (a) *Jun* gene expression, and (b) *Nos-2* gene expression between the two genotypes. Gene expression data obtained was normalized to *Ppia* gene expression.

Table 4.3-Differential gene expression in WT and *Jbo*/+ BMDM on LPS activation in hypoxia and normoxia

Gene	LPS							
	Normoxia				Hypoxia			
	WT 2h	*Jbo*/+ 2h	WT 24 h	*Jbo*/+ 24 h	WT 2h	*Jbo*/+ 2h	WT 24 h	*Jbo*/+ 24 h
Tnf-α	70 (62, 79)	72 (46, 113)	4.9 (4.0, 6.2)	4.8 (4.0, 5.6)	72 (61, 84)	46 (41, 51)	9.1 (8.1, 10.1)	7.3 (6.0, 8.9)
Il-6	332 (308, 358)	334 (216, 517)	1200 (1140, 1250)	1720 (1450, 2040)	1850 (1622, 2110)	1547 (1385, 1729)	756 (696, 821)	911 (758, 1095)
Il-1β	346 (314, 382)	338 (219, 521)	1049 (931, 1183)	1075 (885, 1305)	584 (514, 664)	415 (379, 453)	1267 (1174, 1366)	1208 (1008, 1447)
Vegf	1.9 (1.5, 2.4)	1.7 (1.1, 2.8)	13.3 (10.8, 16.5)	29.1 (25.9, 32.8)	4.9 (3.0, 8.0)	2.9 (2.3, 3.8)	12.1 (10.7, 13.7)	14.9 (11.8, 18.*)
Jun	1.4 (1.2, 1.7)	1.5 (0.9, 2.4)			1.0 (0.7, 1.4)	0.8 (0.6, 1.0)		
Nos-2	37 (28, 48)	32 (18, 57)			297 (233, 378)	287 (230, 358)		

Table 4.3 shows the fold change in treated WT and *Jbo*/+ BMDM observed after LPS activation of *Junbo* BMDM at 2 h and 24 h in normoxia and hypoxia. Values in parenthesis represent RQmin-max values. Coloured numerical values (blue and red) represent significant difference between genotype at that time point (P< 0.05). Numerical figures in blue were not considered biologically relevant due to small differences between the genotypes (< 30%) or did not make a cut-off threshold of 2 fold up/downregulation. Numerical values in red were considered biologically relevant and displayed a strong trend.

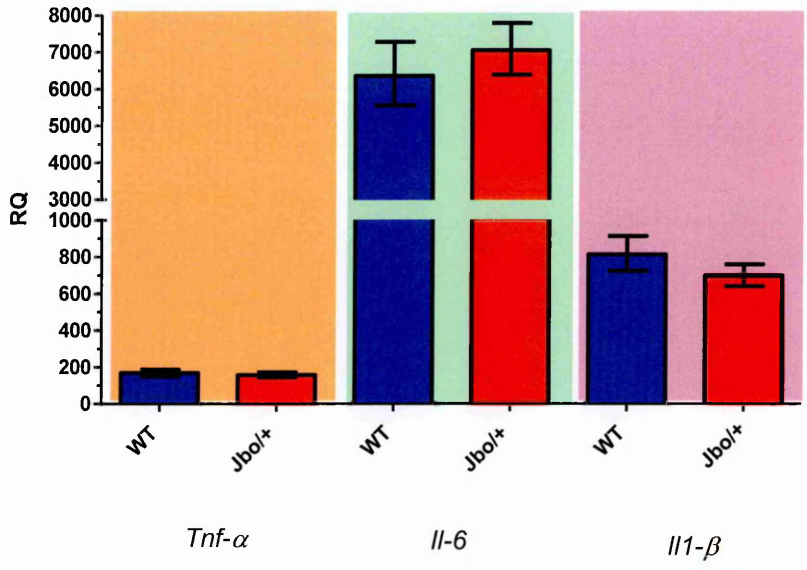
4.4- Hypoxic conditioning increases LPS-responsiveness in both WT and *Jbo/+* BMDM to comparable levels

Having observed genotypic differences on with LPS activation in normoxia and hypoxia, it was important to find out if pre-treatment hypoxia had an effect on classical macrophage activation. It is plausible that prolonged hypoxic conditions prevalent in inflamed *Jbo/+* middle ear might affect inflammatory response of *Jbo/+* macrophages.

This was analyzed by treating BMDM to 22 h hypoxia prior to 2 h LPS treatment in hypoxia. *Tnf-α, Il-1β* and *Il-6* were the three key markers chosen for this study as they are classical LPS activation and inflammatory markers. No significant difference was observed in induction levels of *Tnf-α*, *Il-6*, and *Il-1β* between the genotypes when conditioned with 22 h hypoxia and followed by 2 h LPS activation in hypoxia. However, hypoxic conditioning greatly augmented LPS induced inflammatory gene activation for these genes. *Tnf-α* levels were greatly induced in both genotypes (168.2 fold WT vs. 158.1 fold *Jbo/+* BMDM) with no difference in genotypes (Figure 4.11).

Hypoxia conditioning of BMDM also elevated *Il-6* levels on LPS activation by 6360 fold in WT BMDM and 7050 fold in *Jbo/+* BMDM over genotype-matched untreated control with no significant difference between genotypes (Figure 4.11).

Hypoxia conditioning prior to 2h LPS activation in hypoxia elevated *Il-1β* levels by 813 and 698 fold in WT and *Jbo/+* BMDM respectively but was not significantly different between genotypes.



	WT	Jbo/+
RQ	168	158
RQMin	151	145
RQMax	188	172

WT	Jbo/+
6360	7050
5556	6381
7281	7789

WT	Jbo/+
813	698
724	641
914	760

Figure 5.11- Relative gene expression of 2h hypoxic LPS activation of hypoxia conditioned (22 h) WT and *Jbo/+* BMDM

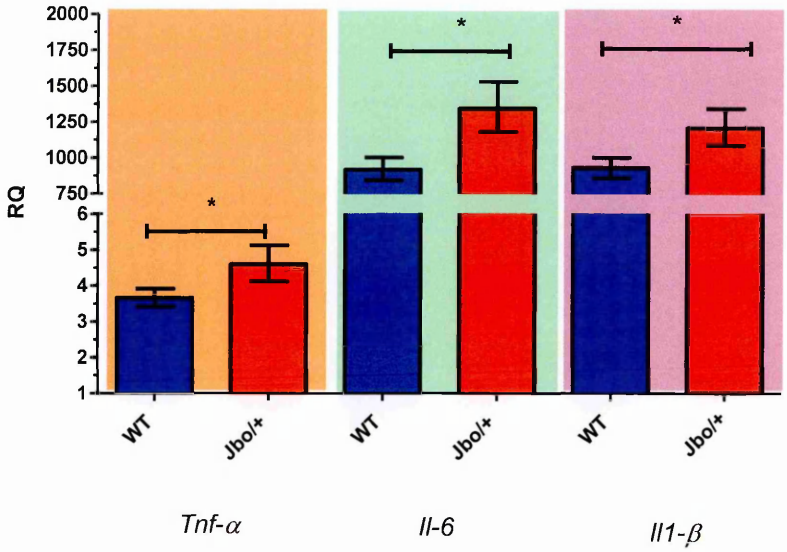
This figure illustrates relative gene expression levels of *Tnf*-α, *Il-6*, *Il-1*β between WT and *Jbo/+* BMDM treated with 250ng/ml LPS in hypoxia for 2h after a 22 h hypoxic pre-treatment conditioning. In this figure, n =6 where each replicate is from individual 6-8 wk old mice. No significant differences were detected between *Jbo/+* and WT.

4.5- Resolution of Inflammation in hypoxia is impaired in *Jbo/+* BMDM

As expression of key inflammation bio-markers *Tnf-α, Il-6, Il-1β* and HIF signalling was dysregulated in *Jbo/+* BMDM on prolonged LPS treatment and with combination of LPS and hypoxia; it was crucial to observe if the resolution of inflammation in hypoxia was compromised in *Jbo/+* BMDM under hypoxic conditions, potentially explaining the persistent inflammation observed in *Jbo*. (Figure 4.12).

However Il-6 levels (918 WT vs. 1343 *Jbo/+* BMDM; $P < 0.05$) and Il-1β levels (929 WT vs. 1207 *Jbo/+* BMDM; $P < 0.05$) were significantly different in *Jbo/+* middle ear. LPS activation results in a downstream cascade of pro-inflammatory signals which might be encountered by *Jbo/+* inflammatory cells during the course of an inflammatory response and I hypothesize that the *Evi-1*^{A2288T} mutation

might interfere with resolution of inflammation in hypoxic conditions in *Jbo/+* mice. For this 2 h LPS activation in normoxia was followed by a hypoxic washout and a 24 h hypoxic incubation. The washout was carried out to remove the LPS stimulation and observe the resolution of inflammation in hypoxic conditions. This resulted in a significant genotypic difference in *Tnf-α* levels between the genotypes (3.7 fold WT vs. 4.5 fold *Jbo/+* BMDM; $P<0.05$). Both *Il-6* and *Il-1β* were significantly greater in *Jbo/+* BMDM than WT BMDM which indicates an aberrant resolution of inflammatory response in *Jbo/+* BMDM in hypoxia (Figure 4.12).



	WT	Jbo/+
RQ	3.7	4.6
RQMin	3.4	4.1
RQMax	3.9	5.1

WT	Jbo/+
918.2	1343.4
842.1	1179.6
1001.0	1529.9

WT	Jbo/+
929.3	1207.5
863.3	1085.4
1000.4	1343.2

Figure 5.12- Resolution of inflammation is impaired in *Jbo/+* BMDM in hypoxia

This figure illustrates relative gene expression levels of *Tnf-α, Il-6, Il-1β* between WT and *Jbo/+* BMDM treated with 250ng/ml LPS in normoxia for 2h followed by a LPS washout and 24 h treatment in hypoxia. Six biological replicates were used for the study, each replicate was obtained from 8d old BMDM from individual 6-8 wk old mice. * $P < 0.05$.

4.6- *Jbo/+* middle ears have high Ox-LDL concentrations

Another key characteristic of inflamed *Jbo/+* ear exudates is the presence of foamy macrophages (Cheeseman et al, 2011; Parkinson et al, 2006). Foamy macrophages are formed by uptake of oxidized low-density lipoporotein (LDL) and subsequent accumulation of cholesterol esters and oxysterols which gives these macrophages a characteristic foamy appearance (Brown et al, 2000; Heinecke et al, 1991). Foamy macrophages are associated with other chronic inflammatory diseases such as artherosclerosis (Steinberg, 1997a; Steinberg, 1997b; Steinberg, 2009). *Jbo/+*middle ear is hypoxic

(Cheeseman et al, 2011). Hypoxia is conducive to oxysterol presence and foam cell formation (Cheeseman et al, 2011; Hulten & Levin, 2009; Parkinson et al, 2006; Rydberg et al, 2004). Ox-LDL assay performed on 12-21 wk old mice (section 2.13.4) revealed that Ox-LDL concentrations in *Jbo/+* ear exudates are 240 folds greater than the Ox-LDL levels in *Jbo/+* or WT serum which are comparable (Figure 4.13). The presence of foamy macrophages and higher amounts of Ox-LDL along with hypoxia might affect the inflammatory response in *Jbo/+* mice. This led to my next hypothesis that the chronic inflammation in *Jbo/+* mice is a result of dysregulated and hypo-responsive foamy macrophages in *Jbo/+* BMDM.

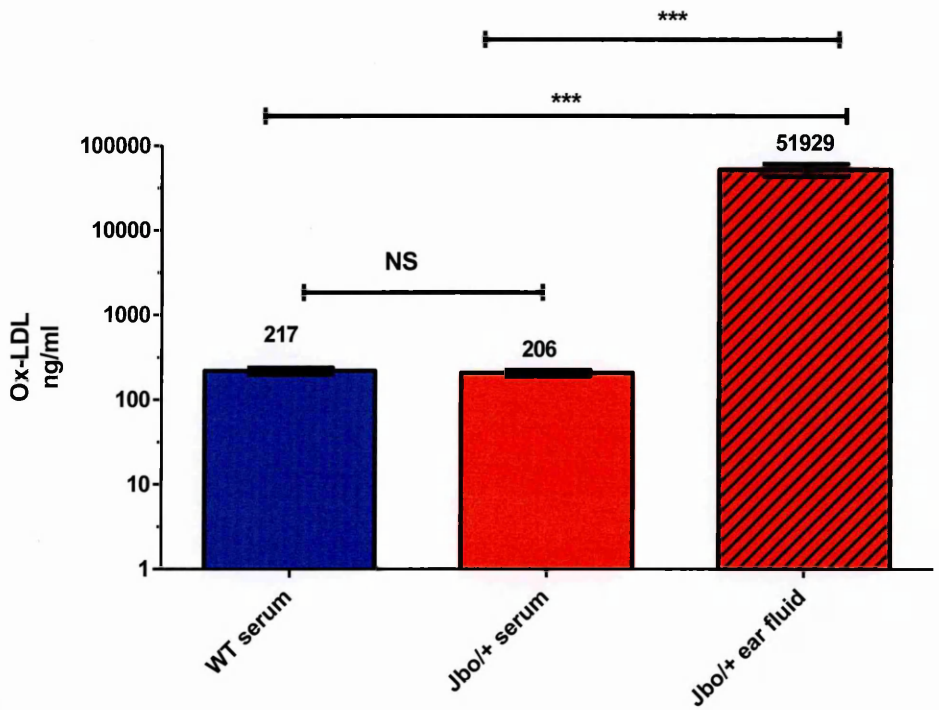


Figure 5.13- *Jbo/+* mice have high Ox-LDL concentration in middle ear

This figure illustrates Ox-LDL concentrations in *Jbo/+* middle ear fluid. Serum samples from WT and *Jbo/+* mice of same cohort were used as control due to absence of fluid in WT middle ear. NS= not significant, ***P <0.0001, n =18 for WT serum, n=15 for *Jbo/+* serum, n=21 for *Jbo/+* ear fluid.

4.7- Foamy *Jbo/+* BMDM under hypoxic conditions have reduced Nos-*2* expression levels

To test hypothesis that Jbo/+ foamy macrophages are hypo-responsive in hypoxic conditions the following experiments were performed. BMDM from *Junbo* mice were subjected to oxysterol treatment in normoxia or hypoxia as well as subjected to an oxysterol washout and LPS activation and 2 h hypoxia exposure to explore if a shift in gene expression profile of Ox-LDL responsive inflammatory genes in *Jbo/+* foamy macrophages results in chronic inflammation observed in *Jbo/+* middle ear. To induce foam cell formation BMDM were treated with oxysterol at a concentration of 5 μg/ml under normoxia for 24 h to load the cells with oxysterol followed by a 12 h oxysterol incubation in normoxia or hypoxia (Figure 4.14) (See section 2.3 for method). A washout of oxysterol was performed to see if the effect of oxysterol was reversed. LPS-responsiveness post oxysterol treatment was also analysed by activating BMDM with 2 h LPS in hypoxia post-washout. Analysis of the response to oxysterol in *Junbo* BMDM was performed by real-time gene expression analysis of an oxysterol responsive gene panel containing cholesterol efflux protein encoding gene *Abca1,* inflammatory marker genes *Tnf-α, Nos-2,* and chemokine *Cxcl2*. Alteration in *Abca1* expression may indicate defective cholesterol efflux and susceptibility to foam cell formation by *Jbo/+* BMDM. *Cxcl2* was chosen as it is a homolog of *IL-8* in mice and a key inflammatory chemokine that attracts macrophages. IL-8 induction in response to oxysterol has been reported in the literature (Liu et al, 1997).

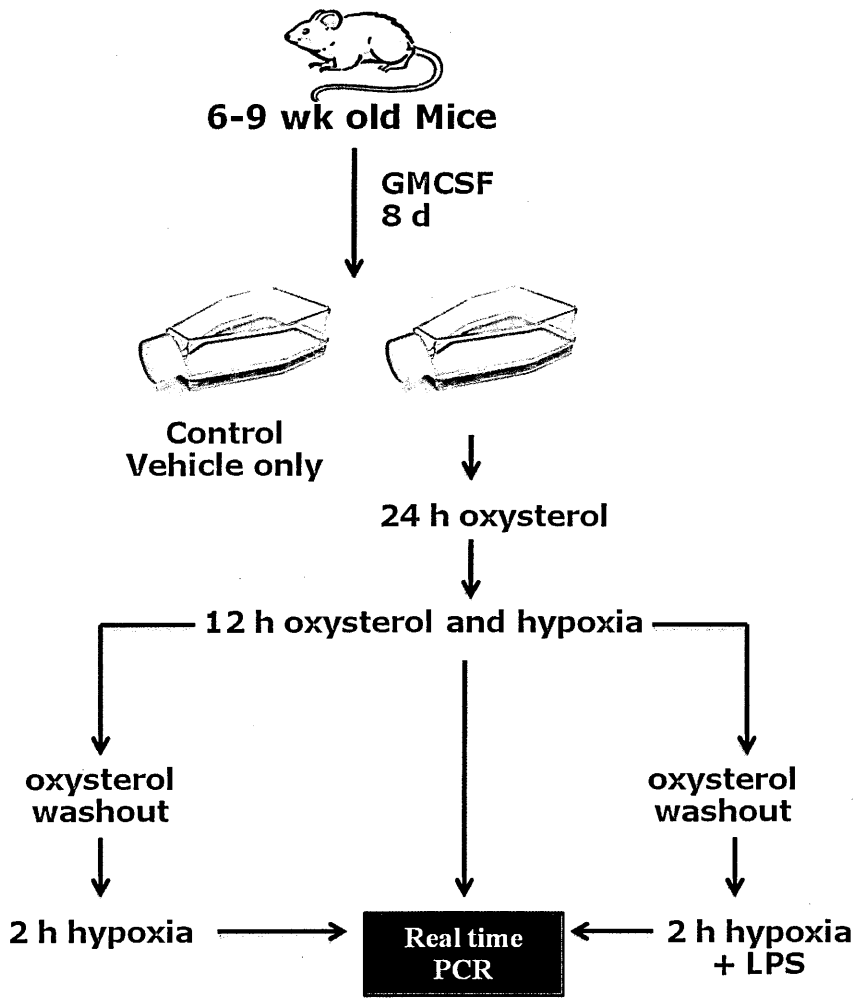


Figure 5.14- Diagrammatic representation of oxysterol studies

BMDM from femurs of 6-9 wk old mice were cultured in GM-CSF. BMDM were treated to 5μg/ml of oxysterol in normoxia for 24 h to lipid load the BMDM. BMDM were then exposed to one of the 4 treatment conditions. Cells were then harvested and analysed using RT-qPCR.

- 12 h oxysterol incubation in normoxia
- 12 h oxysterol incubation in hypoxia
- Oxysterol washout followed by 2 h hypoxia
- Oxysterol washout followed by 2 h hypoxia and LPS

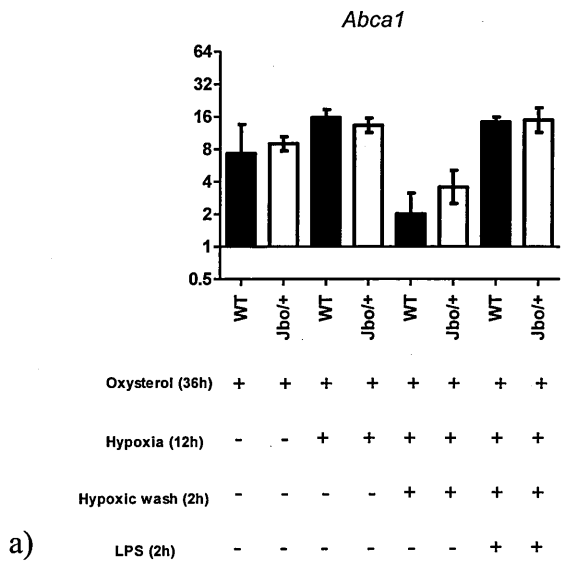
Abca1 expression was upregulated by oxysterol treatment in normoxia (7.3 fold WT vs. 9.0 fold) and hypoxia (15.7 fold WT vs. 13.3 fold *Jbo/+* BMDM) with no significant genotypic difference in its expression (Figure 14.15a). Removal of oxysterol stimulus by washout in hypoxia resulted rapid reduction of induced *Abca1* levels (2 fold in WT vs. 3.6 fold in *Jbo/+* BMDM). 2 h hypoxic LPS activation post-oxysterol treatment and washout resulted in induction of *Abca1* levels (14.3 fold WT vs. 14.9 fold *Jbo/+* BMDM) (Figure14.15a).

Tnf-α expression was induced with oxysterol treatment in normoxia (4.9 fold WT vs. 4.3 fold *Jbo/+*) but not in hypoxia (0.9 fold in WT and *Jbo/+* BMDM). No difference between genotypes was observed with either treatment. *Tnf-α* levels were induced with 2 h hypoxia exposure post-oxysterol treatment and washout (12.0 fold in WT vs. 10.6 fold *Jbo/+* BMDM). Exposure of BMDM to oxysterol reduced LPS responsiveness and reduced *Tnf-α* induction by LPS in hypoxia (22.3 fold WT vs. 19.8 fold *Jbo/+* BMDM) (Figure 14.5b).

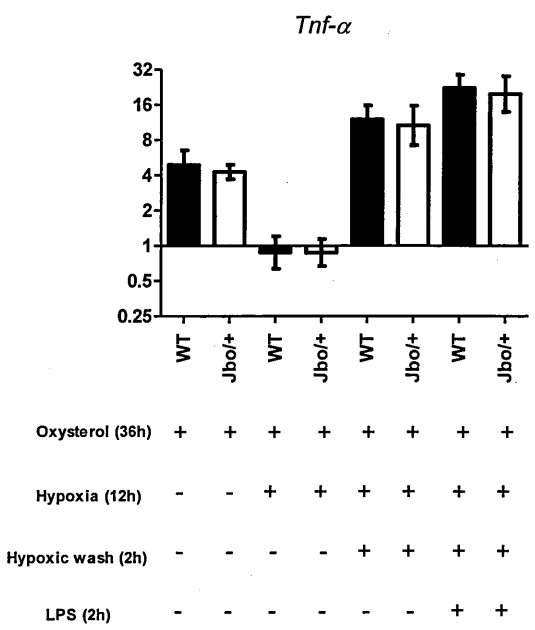
Cxcl2 levels were upregulated by oxysterol treatment in normoxia (2.7 fold WT vs. 2.3 fold *Jbo/+* BMDM) and hypoxia (2.7 fold WT vs. 3.1 fold in *Jbo/+* BMDM) with no significant difference between genotypes. *Cxcl2* levels were downregulated with the removal of oxysterol in both genotypes (0.4 in WT and *Jbo/+* BMDM). Hypoxic LPS activation post oxysterol treatment and washout resulted in *Cxcl2* upregulation (80.1 fold vs. 55.5 fold). However, no significant genotypic difference in *Cxcl2* levels was observed (Figure 14.5c).

Nos-2 expression was elevated by 3.1 fold in WT BMDM vs. 1.3 fold in *Jbo/+* BMDM on oxysterol treatment in normoxia with no significant difference between the genotypes. Oxysterol treatment in hypoxia resulted

in a significant difference in *Nos-2* levels between the genotypes. *Nos-2* levels were 2.5 fold induced in WT vs. 0.6 in *Jbo/+* BMDM ($P < 0.05$). Oxysterol washout and 2 h hypoxia exposure post-treatment increased *Nos-2* to comparable levels (2.8 in WT vs. 3.3 in *Jbo/+* BMDM; $P > 0.05$) LPS responsive *Nos-2* activation in hypoxia was inhibited with oxysterol treatment to comparable levels in both genotypes (4.4 fold in WT vs. 4.6 fold in *Jbo/+* BMDM; $P > 0.05$) (Figure 14.5d).



a)



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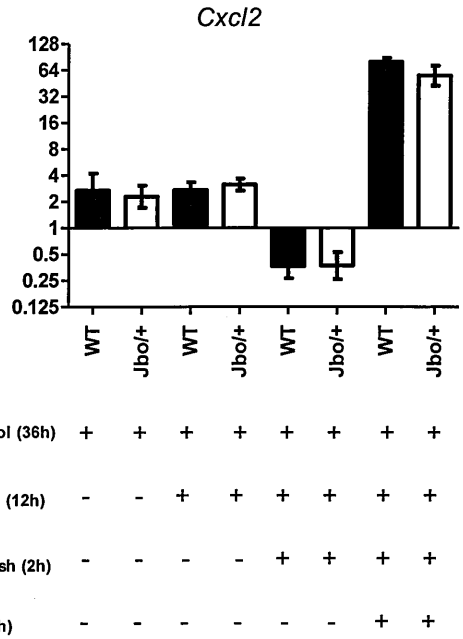

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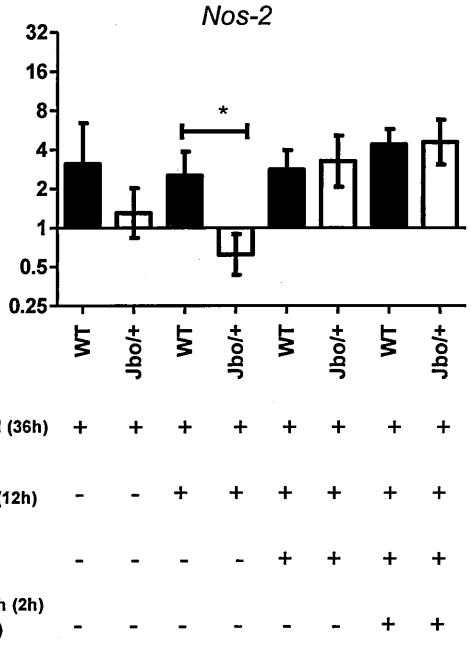

Figure 5.15- Oxysterol treatment modulates gene expression in *Junbo* BMDM

This figure illustrates relative gene expression levels of *Tnf-α, Abca1, Nos2, Cxcl2* between WT and *Jbo/+* BMDM treated with oxysterol in hypoxia and normoxia, n=6 where each replicate was obtained from 8d old BMDM from individual 6-8 wk old mice. *P<0.05.

Table 4.4- Oxysterol treatment modulates gene expression in *Junbo* BMDM

	Oxysterol treatment							
					2 h hypoxic washout		2 h hypoxic washout + LPS	
Gene	Normoxia		Hypoxia		Hypoxia		Hypoxia	
	WT	*Jbo*/+	WT	*Jbo*/+	WT	*Jbo*/+	WT	*Jbo*/+
Cxcl2	2.7 (1.7, 4.2)	2.3 (1.7, 3.1)	2.7 (2.2, 3.3)	3.1 (2.7, 3.7)	0.4 (0.3, 0.5)	0.4 (0.3, 0.5)	80 (72, 89)	55.5 (43, 72)
Nos-2	3.1 (1.5, 6.4)	1.3 (0.8, 2.0)	2.5 (1.6, 3.9)	0.6 (0.4, 0.9)	2.8 (2.0, 3.9)	3.3 (2.1, 5.1)	4.4 (3.4, 5.7)	4.6 (3.1, 6.8)
Tnf-α	4.9 (3.7, 6.5)	4.3 (3.7, 4.9)	0.9 (0.7, 1.1)	0.9 (0.7, 1.1)	12.0 (9.1, 15.8)	10.6 (7.2, 15.7)	22.3 (17.2, 28.8)	19.8 (13.9, 28.1)
Abca1	7.3 (3.9, 13.6)	9.0 (7.7, 10.4)	15.7 (13.2, 18.6)	13.3 (11.4, 15.5)	2.0 (1.3, 3.1)	3.6 (2.5, 5.1)	14.3 (12.9, 15.8)	14.9 (11.5, 19.4)

Table 4.4 shows the fold change in treated WT and *Jbo*/+ BMDM observed after oxysterol treatment in normoxia and hypoxia and 2 h hypoxia or 2 h LPS and hypoxia exposure post-treatment and oxysterol washout. Values in parenthesis represent RQmin-max values. All numerical values were significantly different. Numerical values in blue indicate minor difference in gene expression which was not considered biologically relevant. Numerical values in red indicate a significant change in gene expression >25% between genotypes.

4.7- Discussion

Evi-1^{A2288T} mutation lies in the ZF2, and ZF2 interacting Evi-1 responsive genes *Fos* and *Jun* encode for components of transcriptional factors AP-1. HIF pathway is known to crosstalk with AP-1 (Laderoute, 2005). According to the literature hypoxia increases the gene expression of *Fos* and *Jun* (Ausserer et al, 1994; Laderoute, 2005; Wang et al, 1995). c-*Fos* and c-*Jun* are reportedly a member of early group of genes where their expression is characterized by early induction by stimuli such as hypoxia. It has been reported that hypoxia responsive early induction of *Jun* is independent of Hif-1α but hypoxia responsive late induction on prolonged hypoxia is dependent on Hif-1α and HIF signalling (Laderoute, 2005). Results from c-*Jun* and c-*Fos* expression from hypoxia responsive studies possibly mimicked the above mentioned biphasic activation. 24 h hypoxia saw induction of both *Jun* and *Fos* expression without a significant difference between genotypes (Table 4.2).

A significant difference ($P < 0.05$) at late induction (72 h hypoxia) of *Jun* and *Fos* was observed between genotypes. *Jun* levels were 15% less induced in *Jbo/+* than WT at 72 h hypoxia. Similarly, *Fos* levels were 20% less induced in *Jbo/+* than WT at 72 h hypoxia. However, the difference between the genotypes was < 25%. Also, the individual fold change in either of the genotype did not meet the 2 fold cut-off value (See Table 4.2) so the difference was not arguably biologically relevant.

Relative gene expression of *Hif-1α* and hypoxia responsive genes *Vegf, Glut-1, Phd2,* and *Pai-1* (Murdoch et al, 2005) was also analyzed. *Hif-1α* is not reported to be a hypoxia responsive gene and as expected its gene expression was not affected by hypoxia.

Vegf is involved in angiogenesis and other cellular processes such as NL recruitment, and mast cell activation (Ferrara, 2009). *Vegf* is known to be Hif-1α dependent hypoxia responsive gene (Forsythe et al, 1996). *Vegf* expression was upregulated with hypoxia in a time dependent manner. No significant difference was observed at earlier time point of 24 h hypoxia in *Vegf* levels between genotypes. However, a significant increase ($P < 0.01$) in *Vegf* levels in *Jbo/+* BMDM was observed with further hypoxic exposure of 48 h (46% increase) and 72 h (33% increase). *Vegf* is known to be co-activated by Smad3 and Hif-1α in hypoxic conditions (Sanchez-Elsner et al, 2001) and the differential upreglation of *Vegf* by the *Jbo/+ Evi-1*^{A2288T} mutation suggests the mutation may act via Smad3. Evi-1 is a repressor of Smad3 (Kurokawa et al, 1998b) and a potential Evi-1 loss of function would increase transcriptional activity of Smad3 to increase Vegf expression in hypoxia as was observed. *Vegf* is also reported to be upregulated by just LPS under normoxic conditions (Blouin et al, 2004; Karin, 2006; Sakuta et al, 2001). *Vegf* expression was upregulated with 2h and 24 h LPS treatment in a time-dependent manner in WT and *Jbo/+* BMDM in normoxia. Interestingly, LPS induced *Vegf* activation was 123% higher in *Jbo/+* than WT BMDM at 24 h LPS activation in normoxia ($P < 0.05$). At 24 h LPS and hypoxia treatment the significant difference in *Vegf* levels was absent. However, the trend of higher *Vegf* levels in *Jbo/+* BMDM was still apparent.

Glut-1 is another hypoxia responsive gene which functions as a glucose transporter and is implicated in cell survival (Burke et al, 2003). *Glut-1* expression pattern in hypoxia was similar to *Vegf* pattern in that *Glut-1*

induction with hypoxia was significantly greater in *Jbo/+* BMDM than WT BMDM at 48 h (28%) and 72 h (57%).

Pai-1 is also a hypoxia responsive as well as Smad3 responsive gene (Dennler et al, 1998b; Pinsky et al, 1998). *Pai-1* levels were increased with hypoxia but the levels gradually decreased on prolonged hypoxia. *Pai-1* levels were 21% less induced in *Jbo/+* BMDM than WT BMDM at 24 h ($P < 0.01$). Prolonged hypoxia at 72 h decreased *Pai-1* expression below the basal level but interestingly Pai-1 levels were 40% more in *Jbo/+* than WT ($P < 0.01$). This differential expression of *Pai-1* may be explained by complex regulation of *Pai-1*. *Pai-1* expression is induced by different signalling pathways as *Pai-1* promoter has various cis-regulatory sites such as SP1 site, AP-1 sites, HRE sites and Smad binding sites which indicates complex transcriptional regulation of this gene (Liao et al, 2007; Nagamine, 2008). Increased Vegf levels has been linked to inhibition of *Pai-1* expression and that is one plausible reason for downregulation of *Pai-1* expression with prolonged hypoxia (Mukai et al, 2007). However, is possible that the *Evi-1*^{A2288T} mutation in *Jbo/+* affects *Pai-1* gene expression through another novel pathway.

Phd2 encodes for an enzyme that is involved in hydroxylation and ubiquitination of Hif-1α (Masson et al, 2001). Expression of *Phd2* is known to increase with hypoxia (D'Angelo et al, 2003a; Metzen et al, 2005). Elevated but comparable *Phd2* expression was observed in both genotypes on hypoxic stimulation which indicates a feedback loop to compensate for reduced hydroxylase activity and prepare cells to clear Hif-1α in normoxia (D'Angelo et al, 2003a).

Expression of key inflammatory gene markers *Tnf-α*, *Il-6*, *Il-1β* and *Nos-2* as well as chemokines *Ccl2* and *Cxcl2* were analysed. *Ccl2* levels decreased in response to hypoxia in a time dependent manner (Table 4.3). However, *Jbo/+* BMDM had 28% lower *Ccl2* levels than WT at 24 h hypoxia ($P < 0.05$) (Table 4.3). However, at 72 h *Ccl2* expression was 50% higher in *Jbo/+* at 72 h ($P < 0.05$). Hypoxia is known to downregulate *Ccl2* in macrophages (Bosco et al, 2004a).

Hypoxia decreased level of *Tnf-α* on its own but the levels were comparable in both genotypes. *Tnf-α* is an early LPS responsive gene (Hume et al, 2002; Locati et al, 2002; Yamakawa et al, 1999). As expected *Tnf-α* levels were upregulated with LPS treatment at 2 h and 24 h in both normoxia and hypoxia with a peak time of 2 h. A genotypic difference in *Tnf-α* expression was observed at combination treatment of 2 h LPS and hypoxia where *Jbo/+ Tnf-α* levels were 36% lower than WT BMDM ($P < 0.05$). Although, similar trend was present even with 24 h hypoxia and LPS treatment but it was not significantly different between the genotypes. This suggests that *Tnf-α* expression is dysregulated in hypoxic activated *Jbo/+* BMDM in acute inflammatory conditions.

Il-6 is also a upregulated with LPS in an NFκB dependent manner (Begum et al, 2004; Yang et al, 2010). *Il-6* expression is known to be upregulated with hypoxia (Albina et al, 1995; Matsui et al, 1999). An upregulation of *Il-6* was observed at prolonged hypoxia (72 h) was observed in both genotypes with no significant genotypic difference in its levels. *Il-6* levels were also upregulated by LPS treatment in a time dependent manner in normoxia. Although *Il-6* levels at 2h were comparable between the two genotypes; *Il-6* induction at 24 h LPS activation was 43% increased in *Jbo/+* BMDM than

WT BMDM (P <0.05). This indicated a dysregulation of expression of inflammatory marker Il-6 in activated macrophages at 24 h LPS.

Il-1β gene expression levels are reported to increase with hypoxia (Scannell, 1996). *Il-1β* gene levels were observed to be upregulated at 24 h but the level of induction was comparable in both genotypes. *Il-1β* levels gradually decreased to basal level with further hypoxic exposure. At 48 h hypoxia *Il-1β* levels were 50% reduced in *Jbo/+* BMDM compared to WT (Table 4.3). However, the result was not considered a biologically relevant result as *Il-1β* in both genotypes was below the arbitrary cut-off value. *Il-1β* is also upregulated by LPS (Hume et al, 2002; Locati et al, 2002; Perera et al, 1998). In the LPS activation studies performed for this chapter, *Il-1β* levels were upregulated with LPS activation in a time dependent manner in normoxia and hypoxia (Table 4.3). Similar to Tnf-α, *Il-1β* levels were 30% lower in *Jbo/+* with 2h LPS and hypoxia treatment (P < 0.05). However, this difference was not observed at combined 24 h activation of LPS and hypoxia.

Nos-2 is a gene associated with production of reactive nitrogen species NO (Leone et al, 1991; Palmer et al, 1988). *Nos-2* expression is upregulated by LPS via hypoxia and NF-κB (Angele et al, 1999; Blouin et al, 2004; Heitmeier et al, 1998; Song et al, 2004). *Nos-2* levels were induced with hypoxia at 24 h and gradually declined with further hypoxia exposure (Table 4.2). LPS activation induced *Nos-2* expression in both genotypes in normoxia as well as hypoxia with no significant difference (Table 4.3). Nos-2 expression was synergistically enhanced by hypoxia and LPS activation.

These results validate that HIF signalling in *Jbo/+* BMDM is dysregulated in resting hypoxic BMDM and activated hypoxic BMDM.

Significant differences between genotypes in expression of inflammatory markers were observed in resolution of inflammation studies in which BMDM were activated with LPS for 2 h followed by LPS washout and 24 h hypoxia exposure. *Tnf-α, Il-6, Il-1β* levels were all higher in *Jbo/+* BMDM by 24%, 46% and 30% respectively ($P < 0.05$). These results support the hypothesis that resolution of inflammation is dysregulated in *Jbo/+* BMDM in hypoxic conditions.

Ox-LDL and its gene regulating component oxysterol accumulate in macrophages to form foamy macrophages which are a key feature of ear exudates in *Jbo/+*. My next hypothesis was that *Evi-1*^{A2288T} mutation predisposes *Jbo/+* BMDM to foam cell formation in hypoxia and that *Jbo/+* foamy macrophages have an aberrant transcriptional profile that affects their function and renders them pro-inflammatory.

Oxysterol is known to induce *Abca1* expression (Jiang et al, 2006; Tang et al, 2004). *Abca1* levels were upregulated comparably in both WT BMDM (7.3 fold) and *Jbo/+* BMDM (9 fold) with oxysterol treatment in normoxia.($P > 0.05$). Hypoxia and LPS enhanced the increase in *Abca1* expression despite oxysterol washout. No significant difference in *Abca1* levels between the genotypes was present which suggests cholesterol efflux in *Jbo/+* BMDM was not affected and that *Jbo/+* BMDM. *Abca1* expression in both genotypes was comparable (Table 4.4). This indicated that cholesterol efflux in *Jbo/+* was not affected because of *Evi-1*^{A2288T} mutation and that both WT and *Jbo/+* were equally prone to foam cell formation.

Oxysterols have been shown to increase *IL-8* levels in macrophages which are further enhanced by hypoxia via AP-1 binding (Liu et al, 1997; Rydberg

et al, 2003). *Cxcl2*, also known as *Mip-2,* is the mouse homolog of human *IL-8* and a key inflammatory chemotactic for macrophages. Results from this chapter revealed that hypoxia did not induce *Cxcl2* expression (Table 4.2; Table 4.6). However, oxysterol increased *Cxcl2* expression in both genotypes (Table 4.6) with no significant difference between the genotypes. Removal of oxysterol stimulus by washout in hypoxia decreased *Cxcl2* expression below basal level (Table 4.6) which confirms role of oxysterol in *Cxcl2* induction. Hypoxic oxysterol treatment followed by washout and 2 h LPS and hypoxia exposure induced *Cxcl2* levels by 80 fold in WT and 55 fold in *Jbo/+* BMDM. Although a trend of lower *Cxcl2* levels in *Jbo/+* was apparent with LPS activation of pre-treatment oxysterol incubated BMDM, the difference was not significant.

Oxysterols in foamy macrophages are known to activate nuclear receptors such as LXRs to modulate gene expression (Ghisletti et al, 2007; Janowski et al, 1999). Activation of PPARγ and LXR in cultured macrophages by oxysterol represses expression of inflammatory mediators such as *Nos-2, Tnf-α* in response to LPS stimulation by working as a promoter specific repressor (Castrillo et al, 2003; Ghisletti et al, 2007; Liu & Shuai, 2008; Pascual et al, 2005). *Nos-2* expression via AP-1 and NF-κB is inhibited by PPARγ and LXR activation of oxysterols (Castrillo et al, 2003; Ghisletti et al, 2007; Liu & Shuai, 2008; Pascual et al, 2005). The current study showed oxysterol treatment enhanced *Nos-2* expression under normoxia and hypoxia in WT but *Nos-2* levels in *Jbo/+* BMDM were 76% lower than WT on oxysterol treatment in hypoxia ($P < 0.0.5$) (Table 4.4). Removal of oxysterol stimuli by hypoxic washout increased *Nos-2* levels in *Jbo/+* BMDM to comparable WT levels. LPS stimulation of *Nos-2* was repressed

with oxysterol pre treatment. This suggests that *Nos-2* activation by AP-1 and NF-κB was repressed in both genotypes under conditions of hypoxia and LPS activation by oxysterol activation of LXR (Ghisletti et al, 2007). However, *Nos-2* regulation involves complex interactions between different transcriptional activators such as GATA, CREB, NF-IL6, p53, SP1 etc apart from NF-κB and AP-1 (Pautz et al, 2010). The increase in *Nos-2* expression studies on hypoxic oxysterol treatment may involve an alternate upregulation via oxysterol which seems to be inhibited in *Jbo/+*. Lower NO due to lower *Nos-2* levels might also make *Jbo /+* BMDM prone to bacterial infection as NO is has key anti-bacterial properties (Bogdan et al, 2000).

Tnf-α is also a NF-κB controlled gene and level of *Tnf-α* induction with LPS following oxysterol incubation was reduced due to LXR activation by oxysterol (Ghisletti et al, 2007). However a comparable induction of *Tnf-α* by oxysterols in normoxic resting BMDM from both genotypes was observed, which is attributed to *Tnf-α* being a direct target for LXR in macrophages (Landis et al, 2002). Hypoxia decreased induced *Tnf-α* level back to basal level. *Tnf-α* levels were upregulated again with oxysterol washout suggesting a complex network of *Tnf-α* regulation and that *Tnf-α* via different mechanisms.

Conclusion:

In summary, studies of cellular processes in *Jbo/+* BMDM are pre-disposed to inflammation under hypoxia and activation state due to dysregulated HIF signalling involving pro-angiogenic genes such as *Vegf, Glut-1, Pai-1*; genes involved in inflammation *Il-6, Tnf-α, Il-1β*; and the chemokine *Ccl2*.

Results from this chapter also indicated an impaired resolution of inflammation in *Jbo/+* BMDM under hypoxic conditions. Reduced *Nos-2* expression in *Jbo/+* foamy macrophages suggests the possibility of increased susceptibility of *Jbo/+* BMDM to bacterial infections in hypoxia.

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Chapter 5

6. TGF-β dysregulation in *Junbo* and *Jeff*

5.1- Introduction

TGF-β is a potent immunosuppressive agent produced by a variety of cells including and is involved in a variety of cellular processes (Massague, 1998; Massague et al, 1990).

As previously noted, *Junbo* and *Jeff* are spontaneous chronic OM models generated at MRC Harwell through an ENU mutagenesis programme. *Jbo/+* mice carry a mutation in the trans-activator protein *Evi-1 gene* (Parkinson et al, 2006) and *Jf/+* mice carry a mutation in Fbox protein *Fbxo11* (Hardisty et al, 2003). Both models display phenotype of chronic inflamed middle ear which manifests as conductive deafness. Both the *Evi-1*^{A2288T} mutation in *Junbo* and the *Fbxo11*^{A1472T} mutation in *Jeff* are implicated in TGF-β signalling via their interactions with Smads. Evi-1 is a co-repressor and interacting partner of Smad3. Fbxo11 on the other hand has been shown to interact indirectly with Smad2. Fbxo11 is implicated in stabilization of Smad2 interacting p53 via an unknown mechanism (Tateossian et al, 2009).

I hypothesize that the mutation in *Jeff* and *Junbo* heterozygotes impacts upon TGF-β signalling to result in chronic middle ear inflammation. Cellular hypoxia is a key feature of inflammation in both these mutants and TGF-β is known to interact with the HIF pathway to modulate gene expression of target genes under hypoxia (McMahon et al, 2006; Sanchez-Elsner et al, 2001). Elevated Smad3 target genes like *Vegf* and *Glut-1* have been observed in middle ear fluids from both mutants which hints towards a

role of this ubiquitous pathway in chronic middle ear inflammation (Cheeseman et al, 2011).

To study the effect of how the *Evi-1*^{A2288T} and *Fbxo11*^{A1472T} mutations impact upon TGF-*β* signalling, an *in-vitro* model using BMDM was developed. BMDM were treated with TGF-β under normoxia and hypoxia to uncover the effect of mutations on TGF-β signalling. Dosage optimization for TGF-β was performed on pooled BMDM from 3 WT mice. BMDM was stimulated with TGF-β at 0 pmol, 25 pmol, 50 pmol, 200 pmol for 48 h. FACS analysis was performed for Lectin-like oxidized LDL receptor-1 (Lox-1) expression on BMDM. Lox-1 is a receptor for Ox-LDL uptake expressed on the cell surface of macrophages and is upregulated by TGF-β treatment (Draude & Lorenz, 2000). Maximum response of Lox-1 to TGF-β cwas observed at of 200 pmol. Upregulation of Lox-1 receptor was observed in both WT and *Jbo/+* BMDM at 24 h time-point with 200pmol TGF-β treatment. No significant difference between the two genotypes was observed (Figure 5.1).

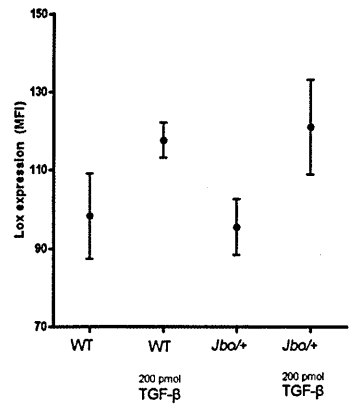


Figure 6.1- Lox-1 is upregulated in both WT and *Jbo/+* BMDM after TGF-β treatment (200 pmol) for 24 h

Result is a mean of 5 individual experiments. Error bars represent the standard deviation. MFI- Mean fluorescence intensity.

5.2- TGF-β (Transforming growth factor-β) pathway is dysregulated *Jbo/+* BMDM under normoxic and hypoxic conditions

Smad3 is a key intracellular effector of TGF-β signalling. It is a transcriptional activator which interacts with other transcriptional activators like Hif-1α and AP-1 transcriptional complex members to co-activate target TGF-β genes such as *TIMP-1, PAI-1, VEGF, FOXP3* (Liberati et al, 1999; Zhang et al, 1998) or co-repress genes such as *NOS-2* (Yoshimura et al). My hypothesis is that the *Evi-1*^{A2288T} mutation dysregulates TGF-β signalling in *Junbo* which results in perpetuation of inflammation. Evi-1 suppresses Smad3 via its C-terminal binding protein(CtBP) binding consensus motif containing repression domain between the 2 zinc finger domains (Izutsu et al, 2001; Kurokawa et al, 1998b). Evi-1 also activates JNK and AP-1 pathways via ZF1 and ZF2 respectively (Kurokawa et al, 1998a; Kurokawa et al, 1998b; Kurokawa et al, 2000). These interactions link Evi-1 to TGF-β signalling pathway, suggesting that the mutation may cause OM through effects on TGF-β signalling. Elevated Smad3 downstream targets such as *Vegf* and *Glut-1* have been observed in the inflammatory cell population containing *Jbo/+* middle ear fluid in comparison to WT serum by RT-qPCR array (Cheeseman et al, 2011) as well as in hypoxic *Jbo/+* BMDM in the preceding chapter. This information provides a crucial link between HIF pathway and TGF-β pathway as *Vegf* is co-activated by Smad3 and Hif-1*α*. Interestingly, elevated *Hif-1α* levels have also been observed in the middle ear of *Jbo/+* mice (Cheeseman et al., 2011). This suggests the possibility that the *Evi-1*^{A2288T} mutation may impact upon TGF-β signalling via the canonical Smad3 pathway. If the *Evi-1*^{A2288T} mutation is a loss of function

then an upregulation of Smad3 activated genes would be observed after TGF-β treatment of *Junbo* BMDM.

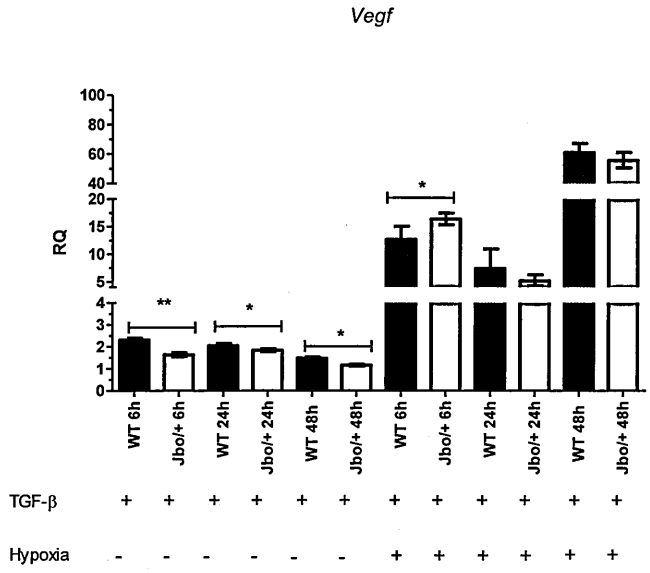
5.2.1- Comparative gene expression analysis of WT and Jbo/+ BMDM with TGF-β treatment in normoxic and hypoxic condition reveals key genotypic differences in *Pai-1, Vegf, Il-1β* and *Il-6* expression

RT-qPCR was used to perform a series of comparative gene expression studies for key TGF-β responsive genes *Vegf, Pai-1, Phd2, Il-1β, Tnf-α, Il-6, c-Jun, c-Fos, Ccl2, Cxcl2)* (Dennler et al, 2002; Fadok et al, 1998b; Feinberg et al, 2004; Jeon et al, 2007; McDonald et al, 1999; Naiki et al, 2005; Schluns et al, 1997; Stroschein et al, 1999; Subramaniam et al, 1995; Viard et al, 1993) as well as *Smad3* and *Hif-1α* (also see section 5.3 for details). A standard dose of 200 pmol was used throughout the experiments and a time course range of 6 h, 24 h and 48 h of TGF-β treatment under both normoxic and hypoxic conditions.

TGF-β treatment in normoxia enhanced *Vegf* expression. *Vegf* levels with 6 h of TGF-β treatment in normoxia were lower in *Jbo/+* BMDM (2.3 fold in WT vs. 1.6 fold in *Jbo/+*; $P < 0.01$) (Figure 5.2a). This trend of lower *Vegf* in *Jbo/+* BMDM with TGF-β treatment was also manifested at 24 h (2.1 fold in WT vs. 1.8 fold in *Jbo/+* BMDM; $P < 0.05$) and 48 h TGF-β treatment in normoxia (1.5 fold WT vs. 1.2 fold in *Jbo/+* BMDM). Exposure of BMDM to hypoxia and TGF-β treatment enhanced induced *Vegf* expression (Figure 5.2a). The elevation in *Vegf* gene expression was most pronounced at 48 h TGF-β (61 fold in WT vs. 55 fold in *Jbo/+* BMDM) but the levels were not significantly different between the genotypes. Exposure of BMDM to hypoxia and TGF-β led to further increment in *Vegf* expression. Interestingly after exposure of BMDM to 6 h

TGF-β and hypoxia, *Vegf* levels in *Jbo/+* were higher than WT (13 fold WT vs. 16 fold in *Jbo/+*; $P < 0.05$). *Vegf* levels were however comparable between both genotypes at 24 h and 48 h of combination treatment and not significantly different.

6 h TGF-β treatment in normoxia upregulated *Pai-1* expression in WT but the *Pai-1* levels in treated *Jbo/+* were lower (2.1 fold WT vs. 1.3 fold *Jbo/+*; $P < 0.01$) (Figure 5.2b). This trend was maintained till 24 h TGF-β treatment in normoxia (but there was no significant difference between the genotypes) and declined back to basal level (1.3 fold WT vs. 1.1 fold *Jbo/+*) in both genotypes by 48 h TGF-β treatment in normoxia. Hypoxia synergistically increased *Pai-1* induction in TGF-β treated BMDM. Interestingly a trend of lower *Pai-1* levels was present in *Jbo/+* (5.7 WT vs. 3.5 fold *Jbo/+*) at 6 h TGF-β and hypoxia exposure, however, the difference between the genotypes was not significant. *Pai-1* levels started declining and were comparable with further hypoxic treatment exposure. *Phd2* expression was not affected by TGF-β treatment under normoxia (Figure 5.2c). However, the addition of hypoxia to the TGF-β treatment saw an upregulation in *Phd2* gene expression which was most pronounced at 48 h of treatment. Although genotypic differences were observed at 6 h (1 fold WT vs. 0.9 fold *Jbo/+*) and 48 h TGF-β treatment (1 fold WT vs. 1.1 fold *Jbo/+*) in normoxia; the differences were too small to be relevant.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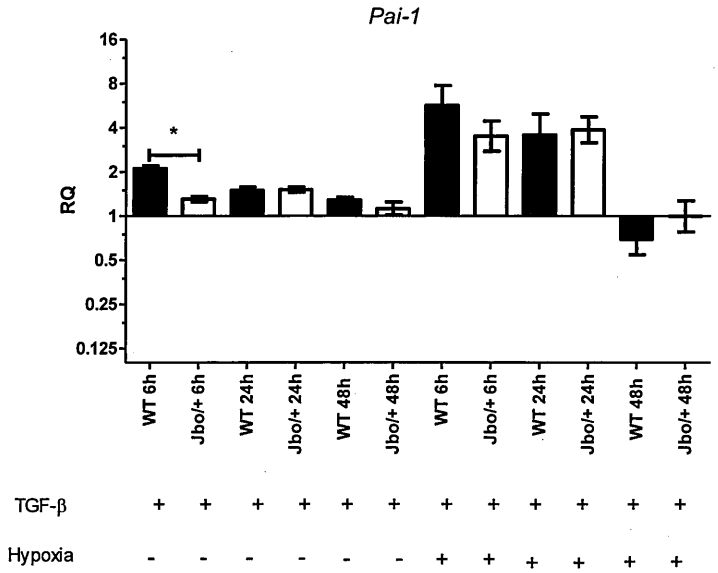
a)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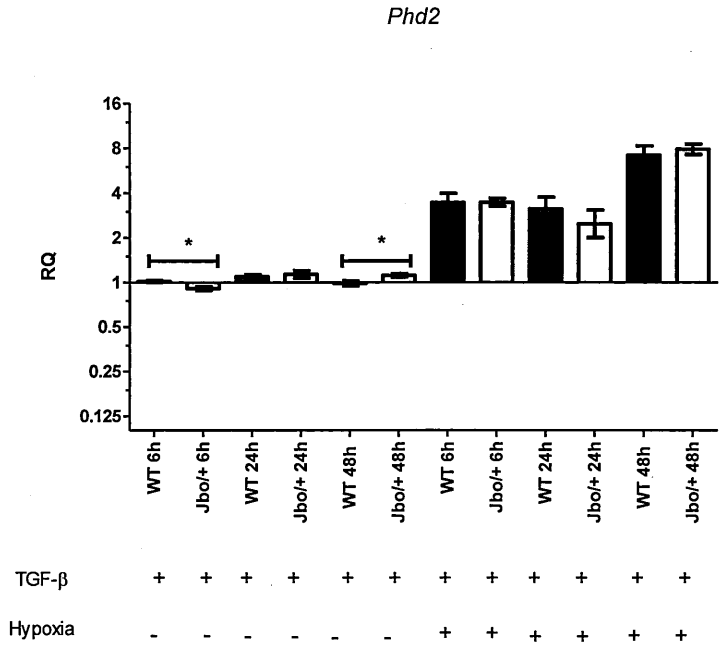
b)



c)

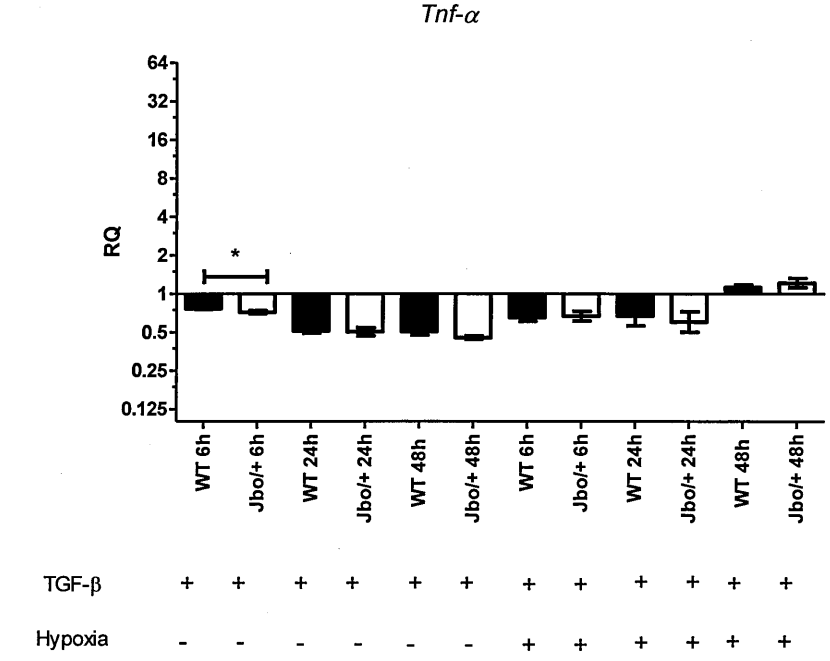
Figure 6.2- Comparative relative gene expression of *Vegf, Pai-1, Phd2* between WT and *Jbo/+* BMDM after TGF-β treatment in normoxia and hypoxia

This figure illustrates relative gene expression levels between WT and *Jbo/+* BMDM treated with 200 pmol TGF-β under normoxia and hypoxia at 6 h, 24 h and 48 h under normoxia and hypoxia. In this figure, n=3 for all TGF-β normoxia studies; n=12 for TGF-β hypoxia studies at 6 h and n=6 for TGF-β hypoxia studies at 24 h and 48 h. Each replicate was obtained from 8d old BMDM from individual 6-8 wk old mice. Figure panels show relative (a) *Vegf* expression, (b) *Pai-1*expression and (c) *Phd2* expression. Statistics were performed as per described in section 2.8 of this thesis. In this figure and all subsequent figures in this chapter, all further figures in this chapter, the bars represents fold increase (RQ) in gene expression observed in 200 pmol TGF-β treated BMDM over the untreated control calibrator. Both WT and *Jbo/+* were calibrated within genotype. Gene expression data obtained was normalized to *Ppia* gene expression. The error bars represent RQ min and RQ max. Blue bars denote WT levels and red bars denote *Jbo/+* levels. * $P < 0.05$

Tnf-α expression was downregulated by TGF-β treatment in normoxia in a time-dependent manner (Figure 5.3a). A 0.5 fold downregulation was observed in both genotypes at 24 h and 48 h TGF-β treatment in normoxia. Hypoxia and TGF-β exposure reversed the normoxic TGF-β responsive downregulation with *Tnf-α* expression coming back to basal level by 48 h hypoxia despite the TGF-β exposure. No difference in expression was observed between the genotypes except at 6 h TGF-β treatment in normoxia. *Jbo/+ Tnf-*α levels were lower in *Jbo/+* BMDM (0hypoxia and TGF-β ($P < 0.01$). *Il-6* expression in *Jbo/+* BMDM was higher in *Jbo/+* BMDM (1.4 WT vs. 4.4 fold *Jbo/+*; $P < 0.01$).

Il-1β expression was downregulated by normoxic TGF-β treatment in a time-dependent manner (Figure 5.3b). Genotypic differences in the form of lower *Il-1* β levels in *Jbo/+* were observed at 6 h (0.8 fold WT vs. 0.7 fold *Jbo/+*; $P < 0.05$) and 48 h TGF-β treatment (0.6 fold WT vs. 0.4 fold *Jbo/+*; $P < 0.01$) in normoxia. TGF-β treatment in hypoxia gradually increased *Il-1β* expression in a time-dependent manner such that a 3.2 fold upregulation was observed in its levels in WT and a 2.5 fold upregulation in *Jbo/+* levels by 48 h but the difference was not significant.

Il-6 gene expression was down-regulated by TGF-β treatment by 0.3 fold in both WT and *Jbo/+* BMDM at 24 h and 48 h of TGF-β treatment in normoxia (Figure 5.3c). TGF-β treatment in hypoxia reversed the downregulation trend in a time-dependent manner. A significant difference between the genotypes was observed under prolonged hypoxia and TGF-β ($P < 0.01$). *Il-6* expression in *Jbo/+* BMDM was higher in *Jbo/+* BMDM (1.4 WT vs. 4.4 fold *Jbo/+*; $P < 0.01$).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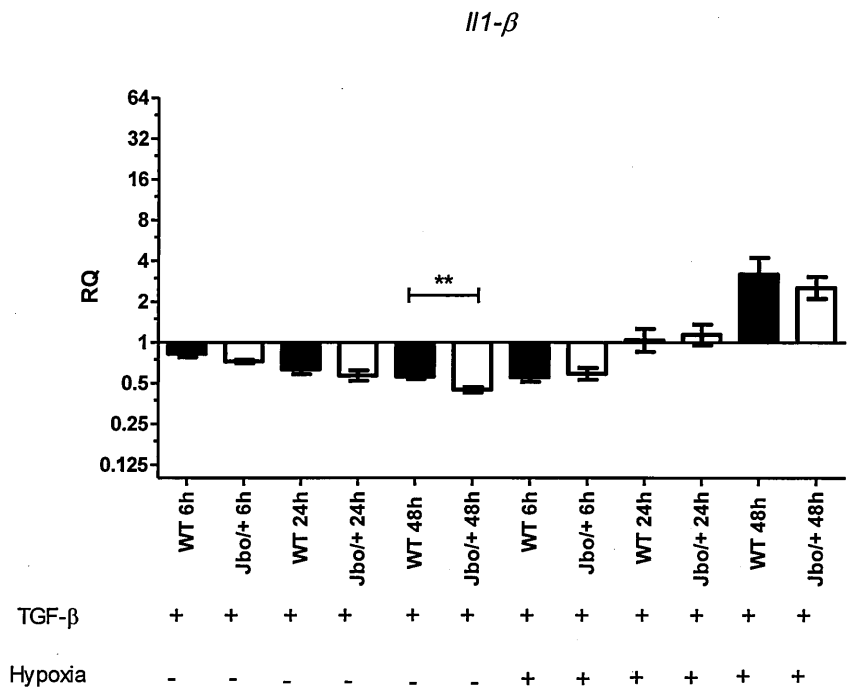
a)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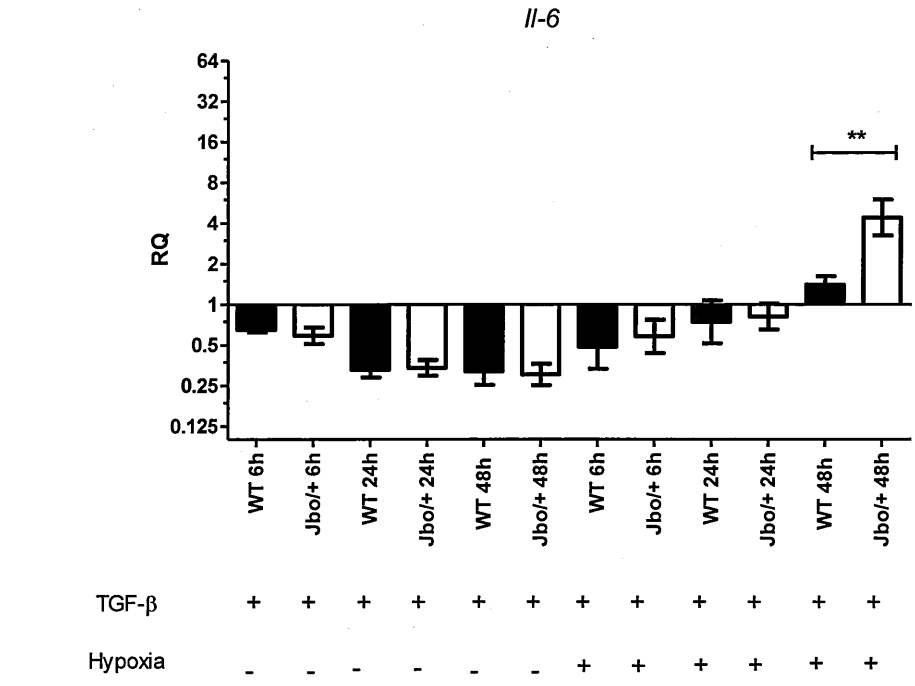


c)

Figure 6.3- Comparative relative gene expression of *Tnf-α, Il-1β, Il-6* between WT and *Jbo/+* BMDM after TGF-β treatment in normoxia and hypoxia

This figure illustrates relative gene expression levels between WT and *Jbo/+* BMDM treated with 200 pmol TGF-β under normoxia and hypoxia at 6 h, 24 h and 48 h under normoxia and hypoxia. In this figure, n=3 for all TGF-β normoxia studies; n=6 for all TGF-β hypoxia studies. Each replicate was obtained from 8d old BMDM from individual 6-8 wk old mice. Figure panels show relative (a) *Tnf-α* expression, (b) *Il-1β* expression and (c) *Il-6* expression. Statistics were performed as per described in section 2.8 of this thesis. Legend is as per in figure 5.2. Blue bars denote WT levels and red bars denote *Jbo/+* levels. ** $P < 0.01$.

5.2.2- Comparative gene expression analysis of WT and *Jbo/+* BMDM with TGF-β treatment in normoxic and hypoxic condition reveals key genotypic differences in *Ccl2, Jun*

Expression of chemokines *Ccl2, Cxcl2* and AP-1 transcription factors *Fos* and *Jun* was checked only at 48 h of TGF-β treatment in hypoxia and normoxia as that seemed the most interesting time-point in previous experiment (Figure 5.4a).

Ccl2 expression was downregulated at 48 h normoxic TGF-β treatment (0.2 fold WT vs. 0.1 *Jbo/+* BMDM) but the difference was not significant (Figure 5.4b). TGF-β treatment in hypoxia revealed a genotypic difference as *Jbo/+ Ccl2* levels were higher than WT (0.3 fold in WT vs. 0.4 fold *Jbo/+* BMDM; $P < 0.05$).

Cxcl2 gene expression was down regulated in both WT and *Jbo/+* (0.3 fold WT vs. 0.2 fold *Jbo/+*) with 48 h TGF-β treatment in normoxia (Figure 5.4c). Hypoxia and TGF-β exposure at 48 h elevated *Cxcl2* levels above baseline (4.2 fold WT vs. 5.6 *Jbo/+*) but the differences were not significant.

No difference in expression of *Fos* expression was observed with the treatment of 48 h TGF-β or 48 h TGF-β and hypoxia.

Jun expression did not change with 48 h TGF-β treatment in normoxia (0.8 fold vs. 0.6 fold *Jbo/+*). With 48 h treatment of hypoxia and TGF-β *Jun* expression in WT was elevated in comparison to *Jbo/+* (1.60 fold WT vs. 1 fold *Jbo/+*; $P < 0.01$) (Figure 5.4d).

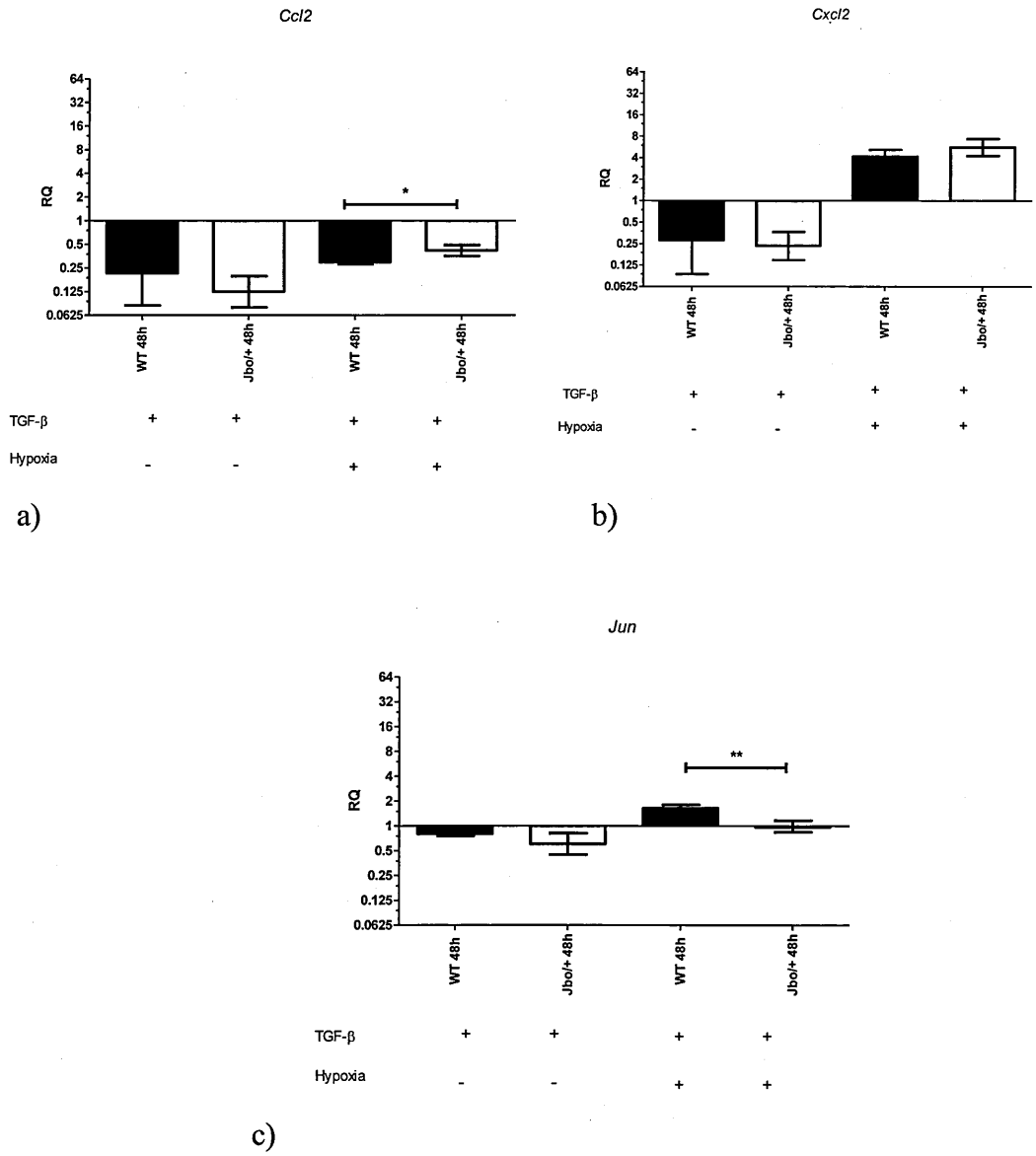
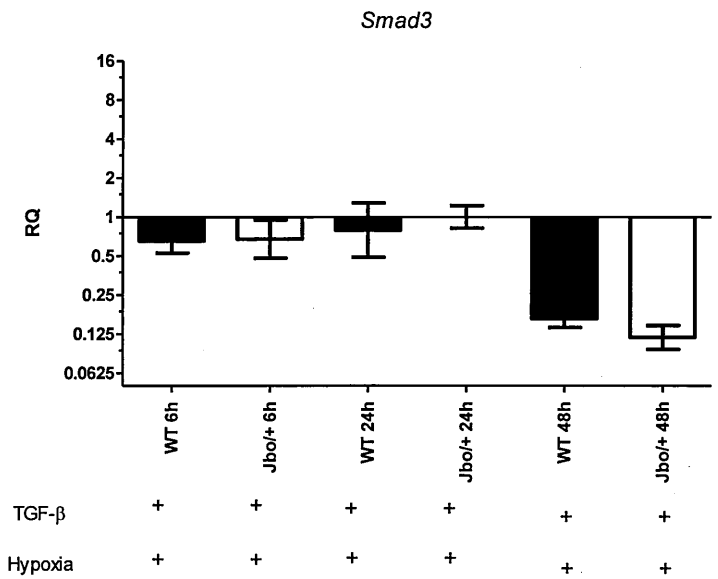


Figure 6.4- Indications of a possible *Ccl2, Jun* dysregulation but absence of dysregulation of *Cxcl2, Fos* at 48 h TGF-β treatment in *Jbo/+* BMDM

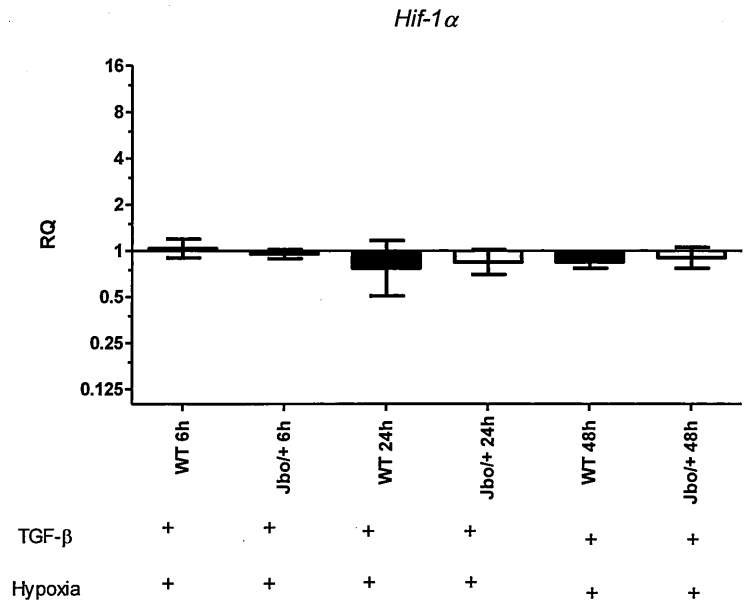
This figure illustrates relative gene expression levels between WT and *Jbo/+* BMDM treated with 200 pmol TGF-β under normoxia and hypoxia at 6 h, 24 h and 48 h under normoxia and hypoxia. 3-6 biological replicates were used as described in figure 5.3. Each replicate was obtained from 8d old BMDM from individual 6-8 wk old mice. Figure panels show relative (a) *Ccl2* expression, (b) *Cxcl2* expression and (c) *Jun* expression. Statistics were performed as per described in section 2.8 of this thesis. Legend is as per figure 5.2. Blue bars denote WT levels and red bars denote *Jbo/+* levels. * $P < 0.05$; ** $P < 0.01$.

5.2.3- Comparative gene expression analysis of WT and *Jbo/+* BMDM with TGF-β treatment in normoxic and hypoxic condition reveals no significant difference in *Smad3* and *Hif-1α*

Next, expression of *Hif-1α* and *Smad3* were checked for the different time-points in TGF-β and hypoxia combined treatment. Expression of *Hif-1α* did not change with TGF-β in normoxia or hypoxia. *Smad3* expression did not change except at 48 h TGF-β treatment in hypoxia where *Smad3* levels downregulated 0.2 fold in WT and 0.1 fold in *Jbo/+* BMDM. There was no significant difference between the genotypes (Figure 5.5a). Hif-1α levels did not change with TGF-β and hypoxia treatment across the time-points (Figure 5.5b).



a)



b)

Figure 6.5- *Smad3* and *Hif-1α* expression is not dysregulated in *Jbo/+* BMDM after TGF-β treatment in hypoxia

This figure illustrates relative *Smad3, Hif-1α* expression levels between WT and *Jbo/+* BMDM treated with 200 pmol TGF-β under hypoxia for 6 h, 24 h, 48 h. 6 biological replicates were used for the study. Each replicate was obtained from 8d old BMDM from individual 6-8 wk old mice. Figure panels show relative (a) *Smad3* expression and (b) *Hif-1α* expression. Statistics were performed as per described in section 2.8 of this thesis. Legend is as per figure 5.2. Blue bars denote WT levels and red bars denote *Jbo/+* levels.

Table 5.1- Genotypic difference in relative gene expression between WT and *Jbo*/+ BMDM after 200 pmol TGF-β treatment in normoxia and hypoxia

Gene	TGF-β											
	Normoxia						Hypoxia					
	WT 6h	*Jbo*/+ 6h	WT 24 h	*Jbo*/+ 24 h	WT 48 h	Jbo/+ 48 h	WT 6h	*Jbo*/+ 6h	WT 24 h	*Jbo*/+ 24 h	WT 48 h	*Jbo*/+ 48 h
Ccl2					0.2 (0.1, 0.5)	0.1 (0.1, 0.2)					0.3 (0.3, 0.3)	0.4 (0.4, 0.5)
Cxcl2					0.3 (0.1, 0.8)	0.2 (0.1, 0.4)					4.2 (3.4, 5.2)	5.5 (4.3, 7.4)
Fos					1.0 (0.9, 1.0)	1.2 (0.9, 1.6)					1.0 (0.9, 1.1)	0.8 (0.7, 1.0)
Hif-1α							1.0 (0.9, 1.2)	1.0 (0.9, 1.0)	0.8 (0.5, 1.2)	0.8 (0.7, 1.0)	0.8 (0.8, 0.9)	0.9 (0.8, 1.1)
Il-1β	0.8 (0.8, 0.9)	0.7 (0.7, 0.7)	0.6 (0.5, 0.6)	0.6 (0.5, 0.6)	0.6 (0.5, 0.6)	0.4 (0.4, 0.5)	0.6 (0.5, 0.6)	0.6 (0.5, 0.6)	1.0 (0.9, 1.3)	1.1 (1.0, 1.4)	3.2 (2.4, 4.2)	2.5 (2.1, 3.1)
Il-6	0.7 (0.6, 0.7)	0.6 (0.5, 0.7)	0.3 (0.3, 0.4)	0.3 (0.3, 0.4)	0.3 (0.3, 0.4)	0.3 (0.3, 0.4)	0.5 (0.3, 0.7)	0.6 (0.4, 0.8)	0.7 (0.5, 1.1)	0.8 (0.7, 1.0)	1.4 (1.2, 1.6)	4.4 (3.2, 6.0)
Jun					0.8 (0.8, 0.8)	0.6 (0.4, 0.8)					1.6 (1.5, 1.8)	1.0 (0.8, 1.2)
Pai-1	2.1 (2.0, 2.2)	1.3 (1.3, 1.4)	1.5 (1.4, 1.6)	1.5 (1.5, 1.6)	1.3 (1.2, 1.3)	1.1 (1.0, 1.2)	5.7 (4.2, 7.7)	3.5 (2.8, 4.4)	3.6 (2.6, 5.0)	3.9 (3.2, 4.7)	0.7 (0.5, 0.9)	1.0 (0.8, 1.3)
Phd2	1.0 (1.0, 1.0)	0.9 (0.9, 0.9)	1.1 (1.1, 1.1)	1.1 (1.1, 1.2)	1.0 (0.9, 1.0)	1.1 (1.1, 1.1)	3.4 (3.0, 4.0)	3.5 (3.3, 3.7)	3.1 (2.6, 3.7)	2.5 (2.0, 3.1)	7.2 (6.2, 8.3)	7.9 (7.3, 8.5)
Smad3							0.7 (0.5, 0.8)	0.7 (0.5, 1.0)	0.8 (0.5, 1.3)	1.0 (0.8, 1.2)	0.2 (0.1, 0.2)	0.1 (0.1, 0.1)
Tnf-α	0.8 (0.8, 0.8)	0.7 (0.7, 0.7)	0.5 (0.5, 0.5)	0.5 (0.5, 0.5)	0.5 (0.5, 0.5)	0.5 (0.4, 0.5)	0.7 (0.6, 0.7)	0.7 (0.6, 0.7)	0.7 (0.6, 0.8)	0.6 (0.5, 0.7)	1.1 (1.1, 1.2)	1.2 (1.1, 1.3)
Vegf	2.3 (2.2, 2.4)	1.6 (1.6, 1.7)	2.1 (2.0, 2.2)	1.8 (1.8, 1.9)	1.5 (1.4, 1.5)	1.2 (1.1, 1.2)	12.6 (10.6, 15.0)	16.4 (15.3, 17.5)	7.4 (5.0, 10.9)	5.1 (4.2, 6.2)	60.9 (55.2, 67.2)	55.5 (50.5, 60.9)

This table shows the summary of the fold change (RQ) in treated WT and *Jbo*/+ BMDM observed after 200 pmol TGF-β treatment of *Junbo* BMDM at 6 h, 24 h, 48 h in normoxia and hypoxia. Values in parenthesis represent RQmin-max values. Coloured numerical values (blue and red) represent significant difference between genotype at that time point (P< 0.05). Numerical figures in blue were not considered biologically relevant due to small differences between the genotypes (< 30%) or did not make a cut-off threshold of 2 fold up/downregulation. Numerical values in red were considered biologically relevant and displayed a strong trend.

5.4- Comparative protein expression analysis between WT and *Jbo/+* BMDM after 48 h TGF-*β* and hypoxia treatment reveals genotypic differences

As RT-qPCR experiments revealed significant differences in pro-inflammatory cytokine *Il-6* gene expression after 48 h TGF-β treatment in hypoxia, it was crucial to see of protein expression of Il-6 or any of the other cytokines was dysregulated in *Jbo/+* BMDM under those conditions. Hence, mouse cytokine arrays (section 2.12) were used to compare relative cytokine and chemokine expression levels between WT and *Jbo/+* BMDM. Cell lysates and supernatant were prepared from *Junbo* BMDM and treated with 48 h hypoxia and TGF-*β* to compare the relative levels of cytokines at the intracellular and extracellular levels by Proteome Profiler Array by R & D Systems. Multiple exposures of the membrane were obtained between 30s and 5 min. Mean pixel density of the signal for each cytokine was obtained by using Chemidoc-It Imaging System and analysed by densitometry with Vision Works software (See Chapter 2 for details). The density signal was compared across both genotypes in untreated and TGF-β treated samples.

122 µg of cell lysate protein and 700 µl of supernatants from 48 h TGF-β and hypoxia treated WT and *Jbo/+* BMDM were loaded on the membrane. 48 h hypoxia and TGF-β was used as this prolonged hypoxia condition would best mimic the micro-environment of the inflamed *Jbo/+* middle ear the most. Also *Il-6* levels under these conditions were 214% higher in *Jbo/+* BMDM than WT (section 5.3). 5 min membrane exposure was used for analysis of cell lysates and 1 min exposure was used for analysis of supernatants after checking for signal-saturation (section 2.12).

For cell lysates from treated BMDM, the array membrane was processed and developed by 5 min X-ray film exposure. Further analysis was performed by estimating level of protein expression by densitometry. The protein data in this thesis is expressed as mean pixel density (section 2.12) after removing background signal. Fold change at basal level was calculated by calibrating untreated *Jbo/+* signal to WT as a control. Differences between treated *Jbo/+* with respect to treated WT were calculated by calibrating treated *Jbo/+* signal to treated WT. Throughout this chapter, difference in cytokine expression is described as fold change in *Jbo/+* over treatment matched WT control.

5.4.1- Proteins detected in cell lysates

Interestingly, out of 40 cytokines on the array, signals for only 4 cytokines (Il-ra, Ccl3, Ccl2, Timp-1) were detected at 48 h hypoxia and TGF-β treated cell lysates from BMDM (Figure 5.6).

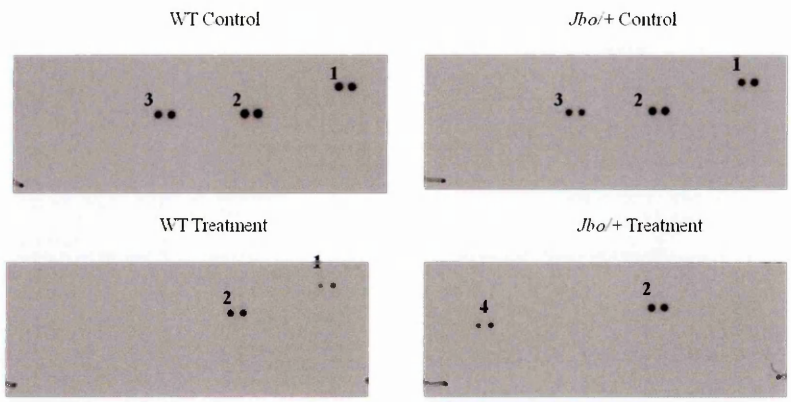
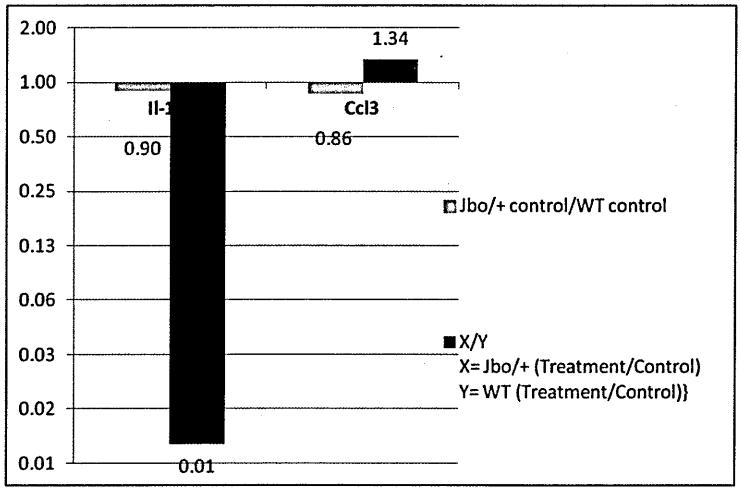


Figure 6.6- Cytokines detected in untreated and 48 h TGF-β and hypoxia teated WT and *Jbo/+* BMDM cell lysates

Untreated and 48 h TGF-β (200 pmol) and hypoxia treated WT and *Jbo/+* BMDM. Each cytokine present was detected as a duplex of dots. Analysis was performed by measuring mean pixel density of each dot on membrane to measure the level of expression of each cytokine/chemokine as described in section 2.12. Cytokine array membrane processed and developed with 5 min exposure to X –ray film. Reference co-ordinates and reference template was used to label cytokines. Cytokines detected are labelled on the figure as follows: (1)- Il-ra; (2) - Ccl3; (3) – Ccl2; (4) – Timp-1.

Il-1ra is a counter-inflammatory cytokine which acts as receptor antagonist for Il-1 cytokines (Dinarello, 2000). Il-ra was present in cell lysates from both untreated WT and *Jbo/+* BMDM controls. Il-1ra levels in the cell lysates were lower in *Jbo/+* BMDM at basal level (0.9 fold WT). 48 h TGF-β and hypoxia treatment reduced expression of Il-1ra in the cell lysates of both genotypes. Il-1ra signal was almost negligible (0.01 fold) in treated *Jbo/+* cell lysates in comparison to treated WT BMDM lysates (Figures 5.6 and 5.7). However, in the supernatants, secreted Il-1ra expression followed a different pattern (figure 5.9; table 5.2, 5.3). Secreted Il-1ra was 0.84 fold compared to WT at basal level. 48 h TGF-β and hypoxia treatment decreased Il-ra levels in supernatant but increased Il-1ra levels in *Jbo/+* supernatant such that after treatment *Jbo/+* Il-1ra levels were 0.96 fold of WT signal.

Ccl3 is a C-C chemokine which acts as chemoattractant for NLs (Moser, 2004). It was present in all 4 cell lysate samples but its expression level was lower in *Jbo/+* BMDM lysate (0.86 fold) than WT BMDM at basal level. Ccl3 levels decreased in both WT and *Jbo/+* BMDM after 48 h hypoxia and TGF-β treatment. However, after treatment the Ccl3 levels in *Jbo/+* BMDM were 1.15 fold higher than WT. Secreted Ccl3 levels in supernatants from control WT and *Jbo/+* BMDM displayed a similar pattern (figure 5.9; table 5.2, 5.3) as Ccl3 levels in *Jbo/+* supernatant were comparable at 1.03 fold.



a)

	WT	Jbo/+	WT Treatment	Jbo/+ Treatment
Il-1ra	39.7391	35.6344	12.5843	0.0913
Ccl3	48.4787	41.8589	29.4129	33.9345

Figure 6.7- Genotypic difference in Il-1ra and Ccl3 expression between WT and *Jbo/+* treated and untreated BMDM

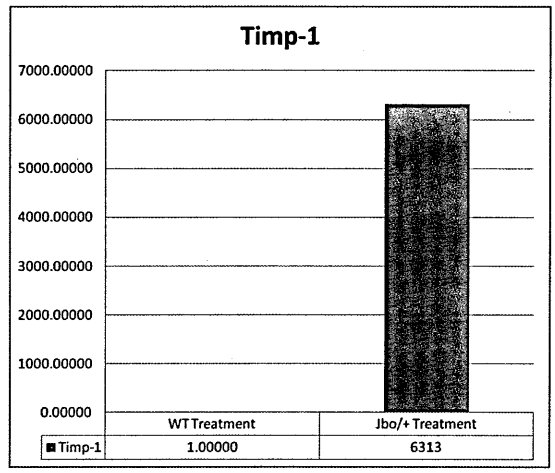
BMDM from WT and *Jbo/+* BMDM were either untreated (used as control) or treated with 48 h of TGF-β (200 pmol) and hypoxia and mean pixel density of each dot on membrane was measured (section 2.12).

a) Relative Il-1ra and Ccl3 levels between WT and *Jbo/+* at basal untreated level and with 48 h of TGF-β and hypoxia treatment. Light purple bars denote difference in relative cytokine expression levels in untreated *Jbo/+* BMDM (Jbo/+ control /WT control. Dark purple bars denote expression levels in 48 h TGF-β and hypoxia treated WT and *Jbo/+* BMDM difference in relative cytokine expression levels after 48 h TGF-β and hypoxia treatment (Jbo/+ treatment/ WT treatment). Relative effect of TGF-β and hypoxia on protein expression levels was calculated for each genotype by calibrating it to the untreated control within genotype. The value obtained for treated *Jbo/+* BMDM was then normalized to treated WT BMDM value. This gave a fold change of cytokine expression level in *Jbo/+* BMDM over WT BMDM after treatment and this fold change was represented graphically. Il-ra level in *Jbo/+* BMDM at basal level was 0.9 fold of WT BMDM. After the treatment, Il-ra level in *Jbo/+* BMDM was .01 fold decline in expression relative to WT.
b) Mean pixel density of untreated and treated WT and *Jbo/+* BMDM for Il-1ra and Ccl2 show that the TGF-β and hypoxia treatment reduced Il-1ra and Ccl2 expression in both genotypes.

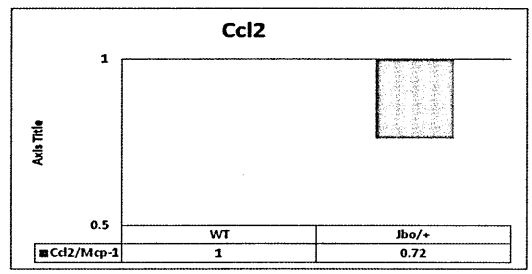
Ccl2 is a monocyte chemoattractant protein which is also a C-C chemokine and is involved in recruitment of NLs as well as monocytes (Moser, 2004). Ccl2 levels were lower in *Jbo/+* BMDM lysates (0.73 fold) than WT BMDM lysate. 48 h hypoxia and TGF-β treatment reduced intracellular Ccl2 levels to below detection levels in both WT and *Jbo/+* BMDM (Figure 5.8). However, in the supernatants, Ccl2 at basal level was again lower in *Jbo/+* supernatant (0.90 fold over WT) at basal level. Ccl2 was detected in supernatant after 48 h hypoxia and TGF-β treatment where Ccl2 levels in treated *Jbo/+* supernatant were 0.95 fold over treated WT supernatant.

Timp-1 is a glycoprotein inhibitor of metalloproteinases (MMPs) (Crocker et al, 2004). Timp-1 levels were below detection level in untreated WT and *Jbo/+* control BMDM lysates in both genotypes. Its expression was massively elevated in treated *Jbo/+* BMDM (6313 fold over treated WT). Secreted Timp-1 was detected in supernatant from all 4 samples (Figure 5.8). Its secretory levels were elevated in *Jbo/ +* untreated supernatant (1.08 fold) compared to WT BMDM and *Jbo/+* treated supernatant (1.09 fold) over corresponding WT calibrator.

a)



b)



	WT	Jbo/+	T Treatme	Jbo/+ Treatment
Ccl2/Mcp-1	36.5721	26.6868	ABSENT	ABSENT
Timp-1	ABSENT	ABSENT	0.0025	15.7827

c)

Figure 6.8- Cytokines detected in untreated and 48 h TGF-β and hypoxia treated WT and *Jbo/+* BMDM cell lysates reveal genotypic difference in Timp-1 and Ccl2 expression

BMDM from WT and *Jbo/+* BMDM were either untreated (used as control) or treated with 48 h of TGF-β (200 pmol) and hypoxia and mean pixel density of each dot on membrane was measured (section 2.12).

a) Timp-1 was below detection levels in untreated control and Ccl2 levels were below detection levels in treated BMDM. So, only treated Timp-1 relative mean pixel density fold change (*Jbo/+* treatment/ WT treatment) was represented graphically for WT and *Jbo/+*. Timp-1 in *Jbo/+* BMDM was 6313 fold over treated WT BMDM.

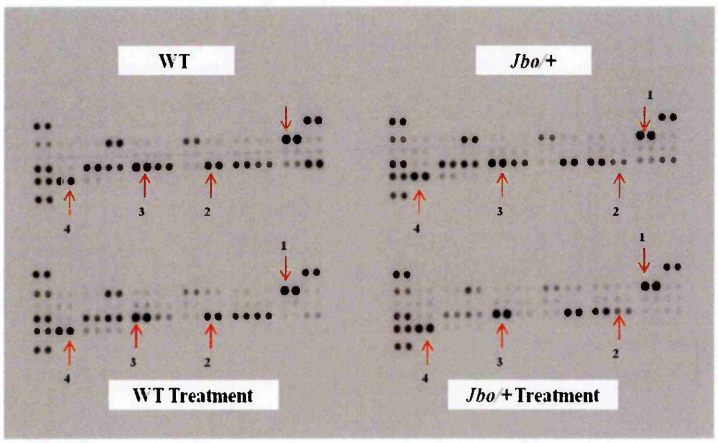
b) Relative mean pixel density fold change for Ccl2 untreated samples (*Jbo/+* control/ WT control) was represented graphically for WT and *Jbo/+*. Ccl2 levels in untreated *Jbo/+* BMDM were 0.72 fold of WT levels.

c) Mean pixel density signal values for Timp-1 and Ccl2 signals.

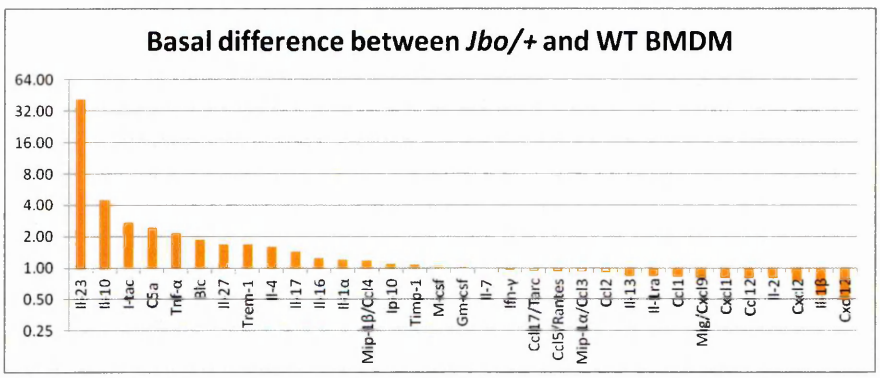
5.4.2- Proteins detected in supernatant

A number of cytokines were detected in supernatant which were below detection levels in the cell lysate. The result is presented in figure 5.9 and tables 5.2 and 5.3.

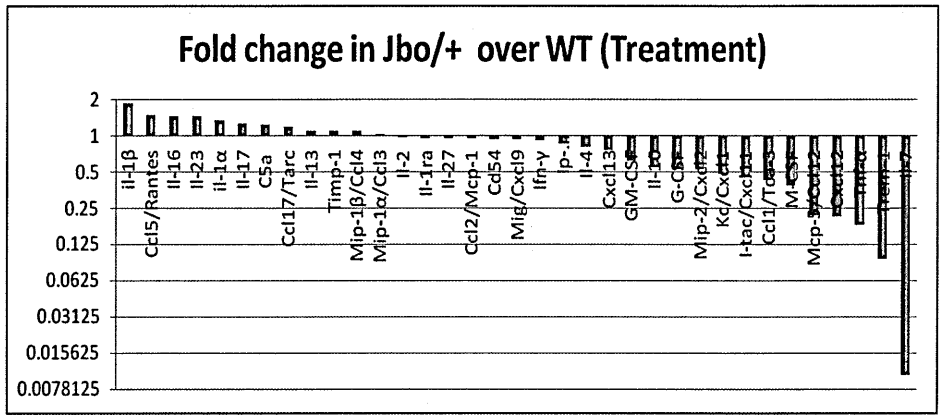
To add to experimental stringency, I have only described cytokines with an arbitrary fold change difference of 2 fold or more between the genotypes at basal level or after treatment in the discussion (section 5.5).



a)



b)



c)

Figure 6.9- Cytokines detected in supernatant from of WT and *Jbo/+* BMDM show TGF-β dysregulation in *Jbo/+* BMDM at basal level and with TGF-β and hypoxic treatment

Relative cytokine levels were obtained using protein profiler array on 600 µl supernatant from untreated and 48 h TGF-β and hypoxia treated WT and *Jbo/+* BMDM. Each cytokine present was detected as a duplex of dots. Analysis was performed by measuring mean pixel density of each dot on membrane to measure the level of expression of each cytokine/chemokine. Vision Works LS software 6.8 by UVP was used for densitometry.

(a) Cytokine array membrane processed and developed with 10 sec exposure to X –ray film. Red arrows donate location of cytokines which were detected in cell lysates and are labelled as follows: (1)- Il-ra; (2) - Ccl3; (3) – Ccl2; (4) – Timp-1

(b) Relative fold change in untreated *Jbo/+* over untreated at basal untreated level. Relative values were obtained for basal level by calibrating *Jbo/+* untreated BMDM to WT BMDM to give a fold change. Relative effect of TGF-β and hypoxia on protein expression levels was calculated for each genotype by calibrating it to the untreated control within genotype and represented in graph. Fold change values are given in Table 5.2.

(c) Relative fold change in *Jbo/+* treated BMDM over WT treated BMDM after 48 h TGF-β and hypoxia treatment . Relative effect of TGF-β and hypoxia on protein expression levels was calculated for *Jbo/+* by calibrating its mean pixel density value to that of treated control. This gave a fold change of cytokine expression level in *Jbo/+* BMDM over WT BMDM after treatment and this fold change was represented graphically. Fold change values after treatment are given in Table 5.3.

Table 5.2- Relative fold change in cytokines at basal level (untreated *Jbo/+* vs. untreated WT)

Cytokine	Fold change in *Jbo/+* over WT at basal level *Jbo/+* control/WT control
Il-23	41.669
Il-10	4.465
I-tac	2.701
C5a	2.430
Tnf-α	2.161
CXCL13	1.860
Il-27	1.671
Trem-1	1.670
Il-4	1.596
Il-17	1.428
Il-16	1.233
Il-1α	1.202
Ccl4	1.165
Ip-10	1.086
Timp-1	1.078
M-csf	1.041
Gm-csf	1.009
Il-7	0.986
Ifn-γ	0.947
Ccl17/Tarc	0.938
Ccl5	0.920
Ccl3	0.917
Ccl2	0.900
Il-13	0.841
Il-1ra	0.835
Ccl1	0.815
Mig/Cxcl9	0.803
Cxcl1	0.801
Ccl12	0.800
Il-2	0.797
Cxcl2	0.746
Il-1β	0.545
Cxcl12	0.493

Values in black represent cytokines upregulated in *Jbo/+* BMDM along with the fold change. Values in green are cytokines which were downregulated in *Jbo/+* BMDM at basal level in untreated controls.

Table 5.3- Relative fold change in treated *Jbo/+* over treated WT BMDM

Cytokine	Fold change -Jbo/+ treated BMDM over treated WT BMDM
Il-1β	1.879
Ccl5/Rantes	1.489
Il-16	1.454
Il-23	1.452
Il-1α	1.354
Il-17	1.271
C5a	1.234
Ccl17/Tarc	1.189
Il-13	1.100
Timp-1	1.093
Mip-1β/Ccl4	1.088
Mip-1α/Ccl3	1.032
Il-2	0.992
Il-1ra	0.957
Il-27	0.953
Ccl2/Mcp-1	0.950
Cd54	0.939
Mig/Cxcl9	0.934
Ifn-γ	0.914
Ip-10/Cxcl10/Crg-2	0.854
Il-4	0.811
Cxcl13	0.763
GM-CSF	0.614
Il-10	0.567
G-CSF	0.525
Mip-2/Cxcl2	0.524
Kc/Cxcl1	0.484
I-tac/Cxcl11	0.458
Ccl1/Tca-3	0.426
M-csf	0.387
Mcp-5/Ccl12	0.219
Cxcl12	0.213
Tnf-α	0.181
Trem-1	0.095
Il-7	0.010

Values in black represent cytokines higher in *Jbo/+* BMDM after treatment. Values in green are cytokines which were lower in *Jbo/+* BMDM after treatment.

5.5- TGF-β pathway is dysregulated in *Jf/+* BMDM in normoxic conditions

Whilst this thesis concentrates on *Jbo/+* mice as a model for OM, limited *Jf/+* mice were available for analysis. *Jeff* is a dominant mouse mutant who displays COME phenotype in humans along with conductive hearing loss (Hardisty-Hughes et al, 2006; Hardisty et al, 2003). The *Jf/+* mouse carries a point mutation in F box gene *Fbxo11* (Hardisty-Hughes et al, 2006). Developmental defects in palate, eyes, lungs in homozygote Jf/Jf mice due to the perturbation of the TGF-β pathway has been observed (Tateossian et al, 2009). Even though the *Jbo/+* phenotype is slightly different from *Jf/+* (OM is less penetrant with serous effusion in comparison to suppurative exudates in *Jbo/+* mice); its hypoxic signalling mechanism seems to acting via Vegf and Hif-1α signalling as the cellular hypoxia in *Jf/+* middle ear has been observed along with upregulated *Vegf* levels in the middle ear effusions (Cheeseman et al, 2011). *Fbxo11* is part of a large family of F-box proteins which function as a part of SKP1-cullin-F-box (SCF) E3 protein ligase complex. These proteins promote proteosomal ubiquitination and degradation by recognizing and binding to phosphorylated proteins (Jin et al, 2004; Kipreos & Pagano, 2000).

Publications suggest that Fbxo11 might be indirectly interacting with Smad2 protein and be involved in TGF-β signalling regulation. Fbxo11 protein acts as a Nedd8-ligase to p53 (Abida et al, 2007). However, a co-immunoprecipitation study performed *Jeff* WT embryo lung extracts failed to reveal any direct interaction between the two proteins (Tateossian et al, 2009). Although *Jf/Jf* homozygotes and *Jf/+* heterozygotes had markedly reduced and intermediate p53 protein levels respectively in comparison to

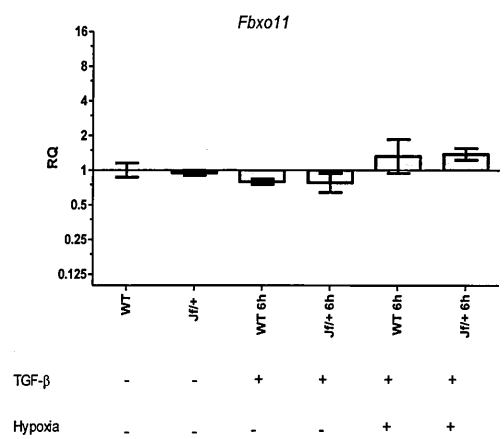
WT. This suggests that Fbxo11 may be required for the stabilization of p53 protein (Tateossian et al, 2009). P53 protein is required for co-activating TGF-β target genes with Smad2 protein (Cordenonsi et al, 2003). Given the above outlined interactions between Fbxo11 protein, p53 and Smad2; my hypothesis is that in *Jf/+* mice, loss of Fbxo11 protein function affects the transcriptional activity of p53 (Figure 1.8). So *Fbxo11*^{A1472T} mutation might result in a decrease of p53 transcriptional targets. Mutant p53 has been shown to result in attenuation of TGF-β responses, reduction in Smad2/3 phosphorylation, inhibition of Smad2/Smad4 complex formation and Smad4 translocation to the nucleus (Kalo et al, 2007). Perturbation in TGF-β signalling might underlie the chronic inflammation observed in *Jf/+* by resulting in an abnormal innate immune response to commensal nasopharyngeal flora resulting in disease. To understand if the *Fbxo11*^{A1472T} mutation affects TGF- β signalling in *Jf/+* gene expression of TGF-β responsive and p53 responsive genes were studied in both WT and *Jf/+* BMDM. RT-qPCR was used to perform a series of comparative gene expression studies for *Fbxo11,*and TGF-β responsive and p53 responsive genes *Pai-1* and *Pai-2*. *Pai-1* and *Pai-2* are genes which encode for plasminogen activator inhibitors which regulate serine proteases and fibrinolysis (Kruithof, 1988; Kruithof, 2008; Kruithof et al, 1995). Pai-1 and Pai-2 induction is often associated with apoptosis and monocyte differentiation and inflammation (Kruithof, 1988; Kruithof, 2008; Kruithof et al, 1995).

No difference between the genotypes was observed in expression of the *Fbxo11* gene; TGF-β treatment did not affect *Fbxo11* expression under normoxia or hypoxia (Figure 5.10a).

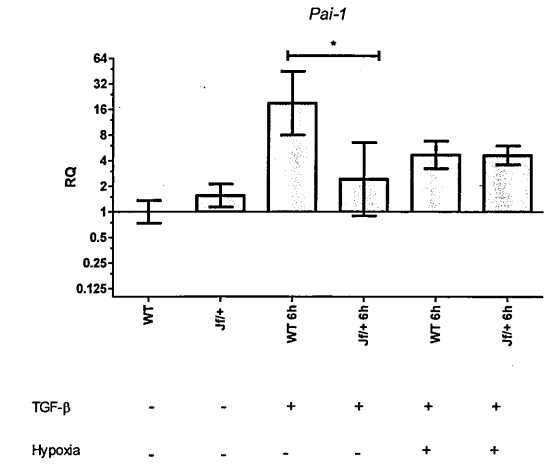
No genotypic difference in *Pai-1* levels (Figure 5.10b) at basal level between WT and *Jf/+* BMDM. *Pai-1* expression was upregulated by TGF-β in both genotypes under normoxia and hypoxia. A significant difference ($P < 0.05$) was observed in *Pai-1* expression levels with 6 h TGF-β treatment in normoxia. 6 h TGF-β treatment in normoxia induced 18.8 fold upregulation in treated WT BMDM vs. 2.4 fold upregulation in treated *Jf/+* BMDM. Addition of hypoxia to the 6 h TGF-treatment induced comparable *Pai-1* expression in both genotypes (4.7 fold in WT vs. 4.6 fold upregulation in *Jf/+* BMDM; $P > 0.05$)

A significant difference in *Pai-2* expression levels (Figure 5.10c) was observed at basal level between WT and *Jf/+* BMDM as *Jf/+ Pai-2* levels were 0.6 fold in comparison to WT BMDM. However this difference was below the threshold cut-off level of 2 fold difference considered to be biologically significant. TGF-β treatment did not affect expression of *Pai-2* in either genotype under normoxia or hypoxia.

a)



b)



c)

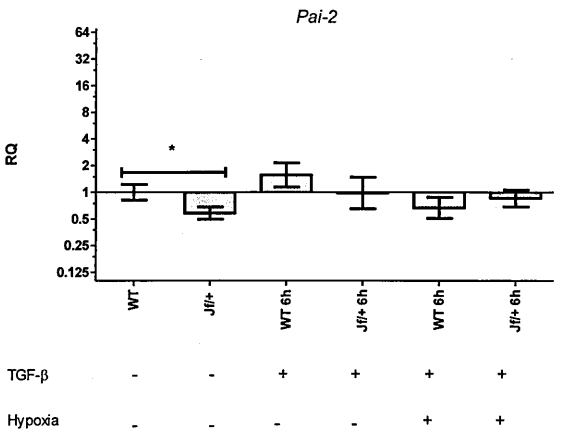


Figure 6.10-Relative gene expression between WT and *Jf/+* BMDM treated with 200 pmol TGF-β for 6 h under normoxia and hypoxia.

This Figure illustrate relative gene expression levels between WT and *Jf/+* BMDM treated with 200pmol TGF-β under normoxia and hypoxia at 6 h under normoxia and hypoxia, n= 9 for TGF-β under normoxia,; n=11 for TGF-β under hypoxia. The bars represents fold increase (RQ) in gene expression observed in 200pmol TGF-β treated BMDM over the untreated control calibrator. Green bars denote WT levels and pink bars denote *Jf/+* levels. Both WT and *Jf/+* were calibrated within genotype. The error bars represent RQ min and RQ max. The y axis is fold change over genotype. Gene expression data obtained was normalized to *Ppia* gene expression. *P < 0.05.

5.6- Discussion

Both *Evi-1* gene and Evi-1 protein are expressed in at comparable levels in WT and *Jbo/+* BMDM, suggesting that the mutation in *Junbo* mice does not affect the transcription or translation of the Evi-1 protein (see chapter 3). Evi-1 is a transcription factor which binds and represses TGF-β effector Smad3 (Kurokawa et al., 1998). Evi-1 also binds c-*fos* promoters to activate JNK and AP-1 pathways via ZF1 and ZF2 respectively (Kurokawa et al, 1998a; Kurokawa et al, 1998b; Kurokawa et al, 2000). This suggests that a mutation in *Evi-1* may impact upon TGF-β signalling. As Smad3 is part of the canonical pathway for TGF-β signalling and if the mutation affects binding of Evi-1 to Smad3 then I hypothesized that elevated levels of Smad3 activated genes and proteins would be present in *Jbo/+* BMDM. HIF pathway and TGF-β cross-talk extensively via Smad3 and AP-1 (See chapter 1). As the inflamed middle ear in *Jbo/+* is hypoxic it was also important to check for TGF-β dysregulation under hypoxia.

TGF-β increased expression of TGF-β/Smad3 responsive genes *Pai-1, Vegf* and decreased expression of pro-inflammatory genes *Tnf-α, Il-1β, Il-6, Ccl2, Cxcl2* under normoxia. Combination of hypoxia and TGF-β led to an additive increase in *Vegf* and *Pai-1* gene expression. However, nosignificant difference in *Vegf* expression in *Junbo* BMDM was observed with TGF-β stimulation under hypoxic conditions. Expression of *Pai-1* was significantly lower in *Jbo/+* BMDM in comparison to treated BMDM by 38% in both normoxia and hypoxia but the difference was significant ($P < 0.05$) only under normoxic conditions.

Phd2 is a hypoxia-responsive gene involved in degradation of Hif-1α subunit under normoxic conditions. Although Phd2 expression has been shown to be downregulated with TGF-β via TGF-β/Smad pathway in

HepG2 cells (McMahon et al, 2006) no effect of TGF-β on Phd2 gene expression in *Jbo/+* BMDM was observed.

TGF-β is a known immunosuppressive agent and regulator of inflammation and wound repair. To further explore TGF-β dysregulation and its effect on inflammatory gene markers, *Il-6, Tnf-α* and *Il-1β* were included in the panel of genes studied. TGF-β has been shown to decrease induction of *Tnf-α* mRNA (Naiki et al, 2005) but TGF-β overexpression studies in lungs have shown it to increase *Tnf-α* mRNA expression (Kang et al, 2007).

In comparative protein expression studies, genotypic differences in Il-1ra were observed. Il-1ra is an anti-inflammatory member of Il-1 family that binds Il-1 receptor without inducing a pro-inflammatory cascade (Arend et al, 1998). Il-ra is released by monocytes, macrophages, neutrophils, keratinocytes and exists in three isoforms, 2 intracellular Il-1ra isoforms icIl-1ra and secreted Il-1ra isoform (Arend & Guthridge, 2000). Although IcIl-1ra and is-Il-1ra can both act as Il-1receptor antagonist, a role of icIl-1ra has also been implicated in antagonizing intracellular signalling of Il-1β and Il-1α (Luheshi et al, 2009). Il-ra level was lower in *Jbo/+* than WT in both BMDM lysates (by 10%) and supernatant (16%). 48 h TGF-β treatment and hypoxia treatment of BMDM decreased Il-1ra levels in both genotypes. However, Il-1ra levels in *Jbo/+* were lower (-99%) after the treatment in BMDM lysates and 4% lower in supernatant. Hypoxia has been reported to decrease IL-1RA expression in human alveolar macrophages (Hempel et al, 1996). On the other hand, TGF-β induces Il-1RA synthesis (Muzio et al, 1994; Yoshimura, 2010). Reduced Il-1ra levels in BMDM lysates indicate predisposition of *Jbo/+* BMDM to chronic inflammation as Il-1ra deficiency in *Il-1ra-/-* mice causes RA, spontaneous arterial inflammation, autoimmunity along with delayed wound healing and augmented pro-

inflammatory Il-1β and Il-6 levels (Horai et al, 2000; Ishida et al, 2006; Nicklin et al, 2000). Polymorphism in *IL-1RA (ILRN*2)* in humans results in impaired *IL-1RA* and augmented *IL-1β* and is associated with several chronic diseases of epithelial origin such as psoriasis, SLE, ulcerative colitis etc. (Arend & Guthridge, 2000; Tarlow et al, 1997; Tountas et al, 1999).

The balance between MMP and its reversible inhibitor TIMPs is a dynamic balance essential for ECM synthesis or degradation (Moali & Hulmes, 2009). MMPs are involved in wound-healing, inflammation and angiogenesis and have been implicated in rheumatoid arthritis (RA) (Nagase et al, 2006). Timp-1 is produced mainly by B cells, mononuclear phagocytes (macrophages, DCs), NLs, epithelial cells and fibroblasts (Verstappen & Von den Hoff, 2006). Timp-1 expression was below detection levels in resting *Jbo/+* and WT BMDM lysates but TGF-β and hypoxia treatment at 48 h increased its expression. *Jbo/+* BMDM levels after treatment were a 6313 fold more than WT in cell lysates. Secreted Timp-1 however was detected at basal level and was 8% more than WT while after treatment secreted Timp-1 was only 9% more in *Jbo/+* than WT. *Timp-1* is reported to be upregulated by TGF-β via a Smad3 dependent pathway in human and mouse fibroblasts (Liberati et al, 1999; Verrecchia et al, 2001; Zhang et al, 1998). Hypoxia is also reported to increase *TIMP-1* expression in monocytes and enhance TGF-mediated amplification of TIMP-1 in fibroblasts (Bosco et al, 2006; Papakonstantinou et al, 2002). IL-6 is reported to induce TIMP-1 expression in synoviocytes and chondrocytes (Silacci et al, 1998). Recently, Timp-1 has been identified as a novel protein regulation resistance to infection as *Timp-1* KO are resistant to infection by *P. aeroguinosa* (Lee et al, 2005). Timp-1 overexpression in fibroblasts is associated with proliferation (Lu et al, 2011). *TIMP-1* increase is associated

with lung cancer, ulcerative colitis, atherosclerosis (Defawe et al, 2003; Fong et al, 1996; Okahara et al, 2005).

Timp-1 is a Smad3 co-activated gene, and Evi-1 is a co-repressor of Smad3 (Kurokawa et al, 1998b). Hence, the higher Timp-1 levels in *Jbo/+* BMDM lysates are suggestive of increased Smad3 activity due to impaired *Evi-1*^{A2288T} function. Higher Timp-1 levels may predispose *Jbo/+* BMDM to delayed wound healing in hypoxia and make those sites pre-disposed to infection as well as result in trapping of macrophages at the site of inflammation due to increased ECM deposition (Bosco et al, 2008).

Il-23 is a pro-inflammatory heterodimeric cytokine made of p40 and p19 and secreted by macrophages and DCs in response to infection usually (Kastelein et al, 2007; Langrish et al, 2004; Oppmann et al, 2000). IL-23 is known to induce IFN-γ production by T-cells and is very important for Th-17 cell development which themselves play a crucial role in inflammation and auto-immune disorders (Liu et al, 2009; Wozniak et al, 2006). High IL-23 levels are associated with inflammatory diseases such as rhinitis and Crohn's diseases (Liu et al, 2010; Yen et al, 2006). *Jbo/+* BMDM supernatant had 4167% higher Il-23 levels than WT at basal level. TGF-β treatment increased Il-23 levels in WT but decreased Il-23 signal in *Jbo/+* supernatant. However, even after 48h TGF-β and hypoxia treatment Il-23 levels in treated *Jbo/+* supernatant were still 45% higher than WT. Whilst LPS is known to increase Il-23 expression, TGF-β is known negatively regulate the p40 Il-23 subunit induction via Smad3 (Ogawa et al, 2008). Interestingly, a role of Smad3 in the p19 Il-23 subunit expression has also been implicated (Al-Salleeh & Petro, 2008). High *Jbo/+* levels of Il-23 may be indicative of predisposition of *Jbo/+* mice to chronic inflammation.

Il-10 is a immunoregulatory cytokine that is produced by a number of cells such as macrophages, B cells, mast cells, epithelial cells, Th1 cells, Th2 cells, Tregs, DCs (Moore et al, 2001; Zhang et al, 2010). It is an inhibitor of antigen presentation and pro-inflammatory cytokines such as Il-1β, Il-6, Tnf-α, Il-12 and inducer of Il-1ra cell (Fiorentino et al, 1991; Mosser & Zhang, 2008b; Opal & Huber, 2000; Rousset et al, 1992). Il-10 has NF-κB promoter (Xu et al, 2002). IL-10 knockout mice are susceptible to infection (Matsukawa, 2007). IL-10 inhibits collagen synthesis and prevents fibrosis caused by Tgf-β. IL -/- mice are prone to fibrosis. (Yamamoto et al, 2001)

A clear association between IL-10 and inflammation susceptibility have been observed (Davidson & Diamond, 2001; Moore et al, 2001; Mosser & Zhang, 2008b; Nathan & Ding, 2010; Zhang et al, 2010). *Il-10* deficiency levels are associated with RA, colitis, enhanced IL-17 response, spontaneous inflammation, deregulated Th1 response, spontaneous IBD development, increased LPS susceptibility which manifests as severe hyper-inflammatory response upon infection with pathogens (Davidson & Diamond, 2001; Deckert et al, 2001; Kuhn et al, 1993; Liu et al, 2011; McKinstry et al, 2009; Nathan & Ding, 2010; Pulendran, 2004; Xu et al, 2009; Zhang et al, 2010). Polymorphisms in *IL-10* are linked to asthma with reduced Il-10 production (Lim et al, 1998). *IL-10* polymorphism is also linked to AOM episode following RSV (Alper et al, 2009). Increased IL-10 levels have been observed in OM fluid in mice and humans (Cheeseman et al, 2011; Zhao et al, 2009). Il-10 levels were 347% higher in *Jbo/+* at basal level but 43% lower in supernatant from *Jbo/+* treated BMDM compared to treated WT. 48 h TGF-β treatment increased Il-10 expression in WT but nominally in *Jbo/+* BMDM. TGF-β is reported to induce *Il-10* mRNA in Th17 cells, antigen presenting cells and rat hepatic stellate cells (Moore et

al, 2001; Wang et al, 1998; Xu et al, 2009). AP-1 is also known to activate *Il-10* transcription in Th2 cell (Rooney et al, 1994). TGF-β increases *Il-10* by activating DNA endogenous promoter (Kitani et al, 2003).TGF-β activates *IL-10* promoter via smad4 in Th1 cells. TGF-β rapidly induces IL-10 secretion via smad-4 binding to IL-10 promoter. It is plausible that *Evi-1*^{A2288T} mutation impairs Il-10 expression via impaired AP-1 activation. However, it is interesting to note that Il-10 levels are higher in *Jbo/+* at basal level but lower under hypoxia and TGF-β treatment.

Inducible T cell α chemoattractant (I-tac) also known as CXCL11 is a cytokine which is known to drive Th1 type inflammatory conditions such as RA and psoriasis. It activates CXCR3 bearing cells such as Th1 cells, B cells, NK cells and has a role in T cell recruitment to the site of inflammation (Petkovic et al, 2004). I-tac gene expression is upregulated by NF-κB (Hinata et al, 2003). I-tac levels were 170% more in *Jbo/+* BMDM at basal level but on 48 h hypoxia and TGF-β treatment, I-tac expression in treated *Jbo/+* BMDM was 83% lower than treated WT BMDM. TGF-β and hypoxia treatment decreased I-tac expression in both genotypes. This again implicates a pro-inflammatory profile of *Jbo/+* at basal level.

The complement system is one of the main effectors of antibody mediated immunity and C5a is released by cleavage of complement system component C5 by serine proteases in activated macrophages and NLs (Amara et al, 2008). C5a functions as a potent chemoattractant with role in eosinophil, NL, monocyte, T lymphocyte recruitment, activation of phagocytic cells, vascular permeability, respiratory burst and adaptive immunity (Guo & Ward, 2005). C5a levels were higher in *Jbo/+* both in untreated and treated supernatant by 140% and 23% respectively. TGF-β and hypoxia treatment increased C5a levels in both genotypes but treatment

induced increment in C5a levels was lower in *Jbo/+*. TGF-β is known to increase C5a mRNA in PANC-I cells (Jazag et al, 2005). This potentially indicates higher C5a levels in *Jbo/+* predispose it to inflammation.

Triggering receptor expressed on myeloid cells-1 (Trem-1) is a cytokine which is expressed by neutrophils and macrophages and serves as an amplifier of the TLR initiated pro-inflammatory response to invading pathogens and PAMPs (Bouchon et al, 2000; Zeng et al, 2007). Trem-1 upregulation is associated with animal models of severe bacterial infection and sepsis (Gibot, 2005; Ho et al, 2008; Zeng et al, 2007). *TREM-1* upregulation is also observed in IBD patients and polymorphisms in *TREM-1* are associated with IBD (Jung et al, 2011). *Trem-1* promoter has NF-κB and AP-1 binding site and is regulated by NF-κB (Zeng et al, 2007). Recently, *TREM-1* has been reported as a hypoxia responsive gene as its upregulation in monocyte derived DCs in inflamed hypoxic joints of children has also been reported (Bosco et al, 2011). All this data suggests that Trem-1 is a classic NF-κB responsive and hypoxia responsive gene. Trem-1 level was observed to be 67% higher in *Jbo/+* BMDM at basal level. TGF-β and hypoxia treatment induced Trem-1 expression in WT BMDM (13.26 fold) but not in *Jbo/+* BMDM (0.75 fold) (Table 5.2). Trem-1 levels were 94.3% lower in treated *Jbo/+* BMDM compared to corresponding WT after 48 h TGF-β and hypoxia treatment (Table 5.3). Increased levels of Trem-1 at basal level indicate a pre-disposition of *Jbo/+* BMDM to inflammation. However, reduced Trem-1 levels on 48 h hypoxia and TGF-β exposure indicate a dysregulated Smad3 and TGF-β signaling. It may also be indicative of dysregulated Ap-1 activation due to *Evi-1*^{A2288T} mutation.

Cxcl13 is a LPS responsive B lymphocyte chemoattractant which is also known to be upregulated in joints of RA mouse models (Fujikado et al, 2006; Mikita et al, 2001). Cxcl13 levels were 86% higher in *Jbo/+* BMDM at basal level but lower by 59% after TGF-β and hypoxia treatment.

M-csf is a growth factor involved in differentiation, survival, proliferation as well as cytokine production of monocytes. M-csf is expressed by endothelial cells, fibroblasts as well as macrophages (Hamilton, 2008). M-csf is also known to promote foam cell formation by upregulating scavenger receptor SR-A and *M-CSF* overexpression has been reported in atherosclerotic plaques (Clinton et al, 1992). *M-csf* deficient *op/op* mice have congenital osteoporosis, neuronal and endocrine deficiencies along with a markedly reduced numbers of tissue macrophages (Gow et al, 2010). TGF-β has been reported to increase M-csf production through a non-Smad3 pathway involving Bmp-2 in mesenchymial cells and osteoblasts (Ghosh-Choudhury et al, 2006; Nelimarkka et al, 1997; Takaishi et al, 1994). M-csf expression is also increased by LPS via NF-κB (Huleihel et al, 1993). M-csf expression was comparable in WT and *Jbo/+* at basal level as *Jbo/+* M-csf levels were only 4.1% higher. However, M-csf levels were 63% lower in *Jbo/+* after treatment. Lower M-csf levels again indicate an over-active Smad3 which might be competitively inhibiting M-csf expression by binding to NF-κB and AP-1 binding site of M-csf promoter.

Ccl12 is a structural and functional homologue of human CCL2 (Sarafi et al, 1997). It is more homologous in function and sequence to human CCL2 than murine Ccl2. Ccl12 functions as a chemoattractant for monocytes. Both *Ccl12* and *Ccl2* have HRE binding site on their promoter and although their expression is upregulated on short hypoxia exposure of up to 6 h; *CCL2* expression is down regulated on longer hypoxia exposure of 18 h (Bosco et

al, 2004b; Mojsilovic-Petrovic et al, 2007). Smad3 is known to downregulate *CCL2* in J774A cells (Feinberg et al, 2004). Due to structural and functional homology between *Ccl12* and *CCL2*, it was of interest to note down-regulation on prolonged hypoxic and TGF-β expression in both genotypes (By 64% in WT vs. 90% in *Jbo/+*) (Table 5.3). Ccl12 expression was 25% lower at basal level in *Jbo/+* BMDM and 56% lower after treatment in comparison to respective treatment matched WT counterpart (Table 5.2, 5.3). This can be implicated to enhanced activity of Smad3 due to *Evi-1*^{A2288T} mutation.

Il-7 functions as a growth factor and an anti-apoptotic factor for T cells and B cells. Il-7 signalling via Il-7r activates Stat 5 pathway as well as PI3K/Akt pathway (Ponchel et al, 2011). It has been reported that Il-7 deficient mice have reduced numbers of T and B cells (Peschon et al, 1994). Although Il-7 levels in WT and *Jbo/+* BMDM are comparable at basal level, Il-7 levels after 48 h TGF-β and hypoxia treatment were 90% lower in *Jbo/+* BMDM compared to WT. TGF-β is known to be a negative regulator of Il-7 protein and hypoxia has been reported to down-regulate IL-7 mRNA (Lu et al, 2009; Tang et al, 1997). Reduced number of lymphocytes is observed in a number of auto-immune diseases such as RA, SLE, MS (Ponchel et al, 2011). Low level of Il-7 in *Jbo/+* BMDM after treatment is indicative of dysregulated TGF-β and HIF signaling which potentially effects T cell survival and differentiation at the site of inflammation.

Dysregulated TGF-β signalling was also observed in BMDM from *Jf/+* mice. As only limited mice were available for studying *Jf/+* mice; a study at 6 h time point was performed to study the effect of *Fbxo11*^{A1472T} mutation on p53, Smad2 and TGF-β responsive genes *Pai-1* and *Pai-2*. It has been reported previously that Fbxo11 mutation in *Jf/+* is implicated in p53

stabilization (Tateossian et al, 2009). P53 is essential for expression of Smad2 responsive genes. Based on these findings my hypothesis was that p53 and TGF-β responsive genes would be expressed at a lower level in *Jf/+* due to a potential TGF-β dysregulation and reduced p53 stabilization. At 6 h TGF-β treatment in normoxia, *Pai-1* levels in *Jf/+* were lower by 87.2% ($P < 0.05$). which validated the hypothesis that *Fbxo11*^{A1472T} mutation affected TGF-β signalling due to potential reduced p53 stabilization. However, additional genes were not added to the panel due to limited amount of time. Although *Pai-2* levels were also lower in *Jf/+* BMDM (at basal level), however the difference was small and did not meet the theshold cut-off point of 2 fold so were not considered biologically relevant.

In conclusion, TGF-β signalling is dysregulated in both *Jbo/+* and *Jf/+* BMDM and this is consistent with perturbation of Smad3 and Smad2. Smad3 has also been known to co-activate *TIMP-1* gene (Liberati et al, 1999; Zhang et al, 1998) and Timp-1 signal was strongly present only in TGF-β and hypoxia treated BMDM lysates. Evi-1 is a co-repressor of Smad3. A loss of function of *Evi-1* would mean increased transcriptional activity of Smad3 which is reflected in increased relative levels of *Il-6*, Timp-1, Il-1β observed in *Jbo/+* BMDM. Together this data is suggestive of a TGF-β dysregulation in *Jbo/+* via canonical Smad pathway. Evi-1 also interacts with AP-1 and JNK pathways to increase *c-Jun* expression. *c-Jun* is downregulated in TGF-β treated *Jbo/+* BMDM which might affect its target genes explaining the down-regulation observed in Pai-1 and other differences observed in supernatants and cell lysates from BMDM suggesting a non-canonical pathway of TGF-β to be dysregulated in *Jbo/+* as well under conditions of hypoxia which might result in exacerbating of inflammation as is observed in *Jbo/+* mice. These differences might explain

the predisposition of *Jbo/+* mice to inflammation. There is a complex level of control by hypoxia exerted on macrophages. The hypoxic microenvironment in inflamed *Jbo/+* middle ear might exacerbate the inflammation effect which may have implications for the histo-pathological changes observed in *Jbo/+* middle ear. Further studies may yield a bigger picture of functional alteration of macrophages in *Junbo* mice which results in perpetual middle ear inflammation.

Chapter 6

7. Discussion

6.1- Background summary

OM is the most common cause of hearing loss and surgery in children (Clarke & De, 2005; Kubba et al, 2000). Over the last few years an increasing number of studies have suggested a strong genetic component to OM (Casselbrant & Mandel, 2001; Casselbrant & Mandel, 2005; Casselbrant et al, 1999; Casselbrant et al, 2009; Kvestad et al, 2004; Kvestad et al, 2006; Rovers et al, 2002). However, our knowledge of the genetic susceptibility factors and pathways underlying OM development and pathogenesis is still limited. Single gene mouse models and bacteria challenged inducible models can play a key role in investigation of cellular and genetic pathways and genes involved in OM. Recently, *Junbo* and *Jeff* mice were generated at MRC Harwell through a large-scale ENU mutagenesis program and were subsequently identified as first non-syndromic spontaneous chronic OM models. *Jbo/+* mice have a mutation in *Evi-1*^{A2288T} (Parkinson et al, 2006). Evi-1 is a transcription factor and a co-repressor of Smad3 (Kurokawa et al, 1998b). Smad3 is a transcriptional activator of genes involved in TGF-β signalling (Jeon et al, 2007; Massague & Chen, 2000; Massague & Wotton, 2000; Sanchez-Elsner et al, 2001; Shi et al, 1998; Yoshimura, 2010). Under hypoxic conditions, Smad3 also co-activates gene expression of hypoxia-responsive genes along with Hif-1α - a transcriptional factor stabilized in hypoxia (Jeon et al, 2007). The HIF pathway cross-talks extensively with NF-κB pathway to upregulate many of its target pro-inflammatory genes (Blouin et al, 2004; Murdoch et al, 2005). *Jf/+* mice have an *Fbxo11*^{A1472T} mutation

in *Fbxo11* (Hardisty et al, 2003; Parkinson et al, 2006). Fbxo11 is implicated in the stabilization of p53 (a transcriptional activator that co-activates the target TGF-β genes with Smad2) (Tateossian et al, 2009).

The OM phenotype in *Jbo/+* mice resembles human phenotype of CSOM with highly suppurative exudates containing high numbers of viable and necrotic NLs, and few foamy macrophages (Cheeseman et al, 2011). Thickening of the bulla lining is also observed in *Jbo/+* inflamed ear although perforation of the tympanic membrane is absent (Parkinson et al, 2006). Interestingly, *Jbo/+* mice also develop OM in germ-free conditions (unpublished data) but a delayed onset of OM is observed which suggests that microbes are not necessary for OM development in *Jbo/+* but do accelerate its progression. The OM phenotype in *Jf/+* is similar to the COME symptoms observed in patients as the middle ear in *Jf/+* contains serous fluid (Cheeseman et al, 2011; Hardisty-Hughes et al, 2006). Although gross OM phenotype is more penetrant in *Jbo/+* than *Jf/+*, both models develop spontaneous OM (Cheeseman et al, 2011). Pathologic hypoxia and upregulation of HIF-signalling pathways has been found in middle ear fluid from both *Jbo/+* and *Jf/+* which indicates a role of HIF pathways in chronic OM pathogenesis. However the *Jbo/+* middle ear was found to be chronically more hypoxic as the inflammatory cells in middle ear exudates, mucosal epithelium, connective tissues were all hypoxic in comparison to *Jf/+* where hypoxia was only observed in inflammatory cells. Recently, a role for HIF-VEGF signalling pathways has been described in OM development in *Jbo/+* and *Jf/+*.

TGF-β signalling has been implicated as a common pathway in development of chronic OM in *Jbo/+* and *Jf/+*. TGF-β signalling is involved in cell development, cell differentiation, proliferation, M2

polarization, wound repair and inhibition of inflammatory cytokine production. TGF-$\beta^{-/-}$ and Smad3$^{-/-}$ mice display chronic inflammation, which further suggests their crucial role in autoimmunity and inflammation. TGF-β interacts with HIF signalling under hypoxic conditions which are reported to be a key pathologic feature of middle ear in *Jbo/+* and *Jf/+* mice. TGF-β also interacts with the HIF pathway and modulates the expression of target genes such as *Timp-1, Pai-1* and *Vegf,* which also depend on Smad interacting partners such as Evi-1 protein for expression (Derynck & Zhang, 2003; Sanchez-Elsner et al, 2001; Yu et al, 2002; Zhang, 2009). The HIF signalling pathway cross-talks extensively with the NF-κB pathway to upregulate many of its target pro-inflammatory genes (Blouin et al, 2004; Murdoch et al, 2005). The mutations in *Jbo/+* and *Jf/+* may impact TGF-β signalling and result in chronic inflammation. Extensive cross-talk exists between TGF-β, HIF pathways and inflammatory signalling pathways via their interaction with NF-κB and AP-1 transcriptional activators. The dysregulated or abrogated HIF or TGF-β pathway can have a profound effect on NF-κB. *Evi-1*^{A2288T} mutation in *Jbo/+* might also affect its interaction with AP-1, as their interaction with the HIF pathway. Dysregulated HIF signalling pathway has been implicated in other chronic inflammatory diseases (Dehne & Brune, 2009; Zinkernagel et al, 2008).

ZF2 of Evi-1 also binds to and raises transcriptional activity of AP-1, another factor which interacts with both HIF and TGF-β signalling (Tanaka et al, 1994); (Laderoute, 2005) and TGF-β signalling (Zhang et al, 1998). In light of this information, a hypothesis was developed that A2288T *Evi-1* mutation in *Jbo/+* mice perpetuates inflammation through a dysregulated HIF pathway and TGF-β signalling pathway.

6.2- Aim

The overall, aim of this thesis was to understand how the *Evi-1*^{A2288T} and *Fbxo11*^{A1472T} mutation contribute to chronic OM development and to identify new mechanisms and genes involved in OM pathogenesis. I proposed TGF-β signalling and HIF signalling as the common pathways implicated in OM in *Jbo/+* and *Jf/+* models. The aim of this thesis was to understand how these mutations (in particular A2288T *Evi-1* mutation) impact upon HIF, TGF-β pathways and affect the innate immune system regulation. Although most of this thesis concentrated on the *Jbo/+* model, a limited number of *Jf/+* mice were available towards the end of this thesis for analysis. However, *Jeff* studies were limited to key experiments examining TGF-β at a single time-point.

Understanding of pathways and mechanisms underlying OM in these models will be useful in discovering novel genes associated with OM development and pathogenesis and development of non-surgical therapeutic measures for management of OM which is the most prevalent cause of deafness and surgery in children of the developed world.

6.3-Summary of key results in this thesis

6.3.1- Global inflammatory cell recruitment is not dysregulated in *Jbo/+* mice

Innate immunity plays a crucial role in development and resolution of inflammation. NLs along with macrophages, DCs, and mast cells are players in innate immunity. However, NLs and macrophages are the key inflammatory cells which orchestrate the initiation and resolution of inflammation. Data from intra-peritoneal thioglycollate elicitation study in this thesis indicated that *Evi-1*^{A2288T} mutation does not impair systemic

recruitment of NLs and macrophages in *Jbo/+* mice. This brought environmental factors such as hypoxia to the forefront of subsequent investigations.

6.3.2- Apoptotic NL percentages in WT and *Jbo/+* are comparable under normoxic and hypoxic conditions

Ear exudates in *Jbo/+* are characterized by NLs and foamy macrophages. At the site of inflammation, NLs are the first inflammatory cells to arrive. Dysregulation in NL apoptosis can lead to non-resolving inflammation due to prolonged release of pro-inflammatory mediators. Data presented in this thesis revealed no significant difference in NLs apoptosis performed with BMDNLs from WT and *Jbo/+* normoxic or hypoxic conditions (Section 3.2.4). BMDNLs were used for NL apoptosis studies as WT *Junbo* mice do not have middle ear fluids to harvest for control NLs and also because the ear-exudates from *Jbo/+* mice are a mixture of viable, apoptotic and necrotic NLs. Most of this thesis henceforth, focussed on macrophages as NLs have a short life-span of 6-12 h (Lee et al, 1993) which made them difficult work with (as apoptotis is underway at the time of their isolation). NLs were also unsuitable for studying cellular processes in OM which would involve longer time-points.

6.3.3- BMDM- A model for studying cellular processes in OM

Macrophages have a longer life-span and are found in middle ear exudates from *Jbo/+* middle ear. Macrophages are involved in initiation, augmentation and resolution of an inflammatory response (Zhang & Mosser, 2008). Macrophages are involved in phagocytosis of infected, apoptotic and necrotic cells, release NL chemoattractants and a plethora of other cytokines depending on stimulatory signal. Macrophages also regulate

adaptive and humoral immunity (Zhang & Mosser, 2008). Dysregulation in any of the key macrophage functions can result in chronic inflammation.

The number of macrophages that can be recovered the from middle ear of mice was too low to study cellular processes in OM. To circumvent this problem, *in vivo* to *in vitro* BMDM model was successfully developed. BMDM model can be used to investigate pathways, genes underlying OM by conventional approaches for studying macrophage activation and stimulation with different treatments. Most of this thesis is based on studies in *Jbo/+* BMDM. However, some studies on *Jf/+* BMDM were also performed due to limited availability of mice and time.

In this work the standard BMDM manipulations were exposure to hypoxia, LPS, oxysterol and TGF-β.. Combination of various treatments was performed to build up model complexity to mimic the inflamed middle-ear micro-environment. Dysregulation in HIF and TGF-β signaling was explored mainly by two techniques. Gene expression studies were performed using RT-qPCR and protein expression studies were performed using western blot and Proteome profiler array (only for TGF-β treatment under prolonged hypoxic conditions) which gave the subsequent key findings.

6.3.4- Expression of *Evi-1* gene and protein is not affected by *Evi-1*^{A2288T} mutation in *Jbo/+* BMDM

Evi-1 protein expression was observed in ear-exudate from *Jbo/+*mice. Gene and protein expression studies on resting, hypoxic and activated BMDM from *Junbo* mice also did not reveal any difference in Evi-1 expression between WT and *Jbo/+* genotypes. This is important because it

indicated that the *Evi-1*^{A2288T} mutation does not affect expression of Evi-1 but rather the mutation may impact upon the function of Evi-1.

Evi-1 expression is reported to be increased on activation with LPS at early time point of 2 h (Wells et al, 2003). Data from this thesis reveals a comparable 15% increase in Evi-1 protein expression with LPS activation at 2 h in both genotypes.

Some of the interesting findings that came out of this work were the hypoxia-responsiveness of *Evi-1* and *Map3k14,* and *Gata-2*.Prolonged hypoxia (72 h) resulted in a 70% downregulation of *Evi-1* in both genotypes. This downregulation was also reflected in time-dependent downregulation of Evi-1 responsive genes *Map3k14* and *Gata-2* at 48 h and 72 h hypoxia. Maximum downregulation was observed for both *Map3k14* and *Gata-2* at 72 h hypoxia exposure (60% downregulation). This is a first time evidence of hypoxia responsiveness of *Gata-2*, *Map3k14* or *Evi-1*. Chronic hypoxia is a feature of chronic inflammation. Evi-1 is a repressor of Smad3 (Kurokawa et al, 1998b). This may potentially serve to enhance HIF signalling in prolonged hypoxia as Evi-1 is a co-repressor of Smad3 (Kurokawa et al, 1998b) which co-activates gene expression of hypoxia-responsive genes such as Vegf (Jeon et al, 2007). Which means that at hypoxia-rich sites *Evi-1* downregulation may potentially result in reduced repression of Smad3 leading to enhanced Smad3 transcriptional activity.

6.3.5- *Evi-1*^{A2288T} mutation in *Jbo/+* perpetuates inflammation and angiogenesis under prolonged hypoxic conditions- Role for dysregulated expression of *Vegf, Glut-1, Pai-1* and *Ccl2*

Throughout this thesis, I have focused only genes which are significantly different between the genotypes ($P < 0.05$) and with more than 30%

difference in relative expression levels and only those genes were considered potentially biologically relevant. Also, only a 2 fold or more difference in a gene expression after treatment was considered as a true difference in expression due to treatment. As a result, it is possible that other genes showing smaller differences might have been overlooked in this analysis.

In hypoxia studies performed in this work, *Jbo/+* BMDM were found to have higher levels of *Vegf, Glut-1* expression on prolonged hypoxia.. *Ccl2* and *Pai-1* were downregulated below basal level on prolonged hypoxia at 72 h but the levels were higher in *Jbo/+* compared to WT.

Vegf is a key angiogenic mediator (Ferrara, 2009) which was upregulated in hypoxic *Junbo* BMDM in time dependent manner during prolonged hypoxia. *Vegf* is known to be Hif-1α dependent hypoxia responsive gene which is co-activated by Smad3 and Hif-1α under hypoxic conditions (Forsythe et al, 1996; Jeon et al, 2007; Sanchez-Elsner et al, 2001). *Vegf* levels were 46% and 33% higher in prolonged hypoxic conditions of 48 h and 72 h respectively ($P < 0.01$). Vascular changes and *Vegf* upregulation has been linked to a number of chronic inflammatory diseases and increased *Vegf* levels have been observed in diseases such as rheumatoid arthritis, inflammatory bowel disease (IBD), diabetic retinopathy (Angelo & Kurzrock, 2007). *Vegf* expression is associated with increased recruitment of NLs, macrophages and increased mast cell activation (Angelo & Kurzrock, 2007; Canavese et al, 2010; Detmar et al, 1998). Angiogenesis is required for maintenance of any chronic inflammatory condition as *Vegf* acts through multiple signalling pathways to stimulate cell proliferation, cellular migration and recruitment (Teige et al, 2009; Walshe et al, 2009).

Glut-1 encodes for a transmembrane glucose transporter protein whose transcription is regulated by oncogenes, growth factor and hypoxia (Burke et al, 2003; Ohba et al, 2009; Wenger et al, 2005). *Glut-1* expression pattern in hypoxia treated BMDM was similar to *Vegf* pattern. *Glut-1* expression was significantly greater in *Jbo/+* BMDM than WT BMDM after treatment with hypoxia at both 48h and 72h. *Glut-1* levels were 28% and 57% more in *Jbo/+* BMDM at 48 h and 72 h hypoxia time points. Increased *Glut-1* expression is linked with tumour angiogenesis and with various carcinomas (Ohba et al, 2009). Overexpression of *Glut-1* in vascular smooth muscle cells (VSMCs) is associated with enhanced glucose uptake, increased recruitment of NLs, increased influx of glucose promotes inflammatory signalling pathways and inflammation in response to vascular injury in Sm22α-*Glut-1* transgenic mice (Adhikari et al, 2011). VSMC hypertrophy is also associated with *Glut-1* overexpression and is attributed to TGF-β-Smad3 pathway as increased phosphor-Smad2/3 staining is seen in VSMCs of Sm22α*Glut-1* mice (Adhikari et al, 2011).

Pai-1 is an inhibitor of tissue plasminogen activator and urokinase-type plasminogen activator (Kruithof, 1988). *Pai-1* is involved in fibrinolysis and is associated delayed tissue repair, inhibition of cell adhesion and is upregulated in inflammation and following bacterial infection (Kohler & Grant, 2000; Lim et al, 2011; Stefansson & Lawrence, 1996). *Pai-1* is a hypoxia responsive gene (Pinsky et al, 1998). *Pai-1* levels were increased with hypoxia in BMDM but the levels gradually decreased on prolonged hypoxia. Prolonged 72 h hypoxia decreased *Pai-1* expression below the basal level but *Pai-1* levels were 40% higher in *Jbo/+* BMDM. This suggests that *Pai-1* expression in *Jbo/+* was less responsive to hypoxia. This was a very interesting result as *Pai-1* is a hypoxia responsive gene (Liao et

al, 2007; Pinsky et al, 1998) as well as Smad3 responsive gene (Dennler et al, 1998b; Stroschein et al, 1999).

Ccl2, also known as Mcp-1, is a chemokine which is the homolog of human CCL2. It has been shown to be important in recruitment and activation of monocytes, T cells, mast cells, basophils as well as wound repair (Ferreira et al, 2005). *Ccl2* levels were 50% higher in *Jbo/+* compared to WT BMDM. *Ccl2* is reported to be downregulated by hypoxia as well as Smad3 (Bosco et al, 2004b; Bosco et al, 2006; Feinberg et al, 2004). So potentially, loss of function of A2288T *Evi-1* repression of Smad3 would result in lower *Ccl2* levels in *Jbo/+*. This was observed at 24 h hypoxia ($P < 0.05$) but the difference between the genotypes was below the set arbitrary cut-off of 30%. It could be that at prolonged hypoxia *Ccl2* levels are regulated by other regulatory pathways such as AP-1. Higher *Ccl2* levels on prolonged hypoxia can potentially favour chronic inflammation via increased macrophage mediated NL recruitment at the site of inflammation.

To summarise, Smad3 is implicated in induction of expression of *Vegf, Glut-1* and *Pai-1* and downregulation of *Ccl2* (Adhikari et al, 2011; Dennler et al, 1998a; Feinberg et al, 2004; Sanchez-Elsner et al, 2001). Evi-1 is a co-repressor of Smad3 and hypothetical loss of function of A2288T *Evi-1* would result in enhanced Smad3 activity. *Vegf* is a classic example of gene co-activated by Smad3. Higher levels of *Vegf*, and other Smad3 and hypoxia responsive genes such as *Glut-1* and *Pai-1* were observed in *Jbo/+* BMDM under prolonged hypoxia (Figure 6.1). This indicates a potential loss of function as a result of theA2288T *Evi-1* mutation. Increased levels of *Vegf, Glut-1, Pai-1* and *Ccl2* in *Jbo/+* BMDM in prolonged hypoxia can also potentially exacerbate inflammation by increasing angiogenesis, NL

infiltration, monocyte accumulation, hypertrophy, fibrin accumulation and impaired wound healing.

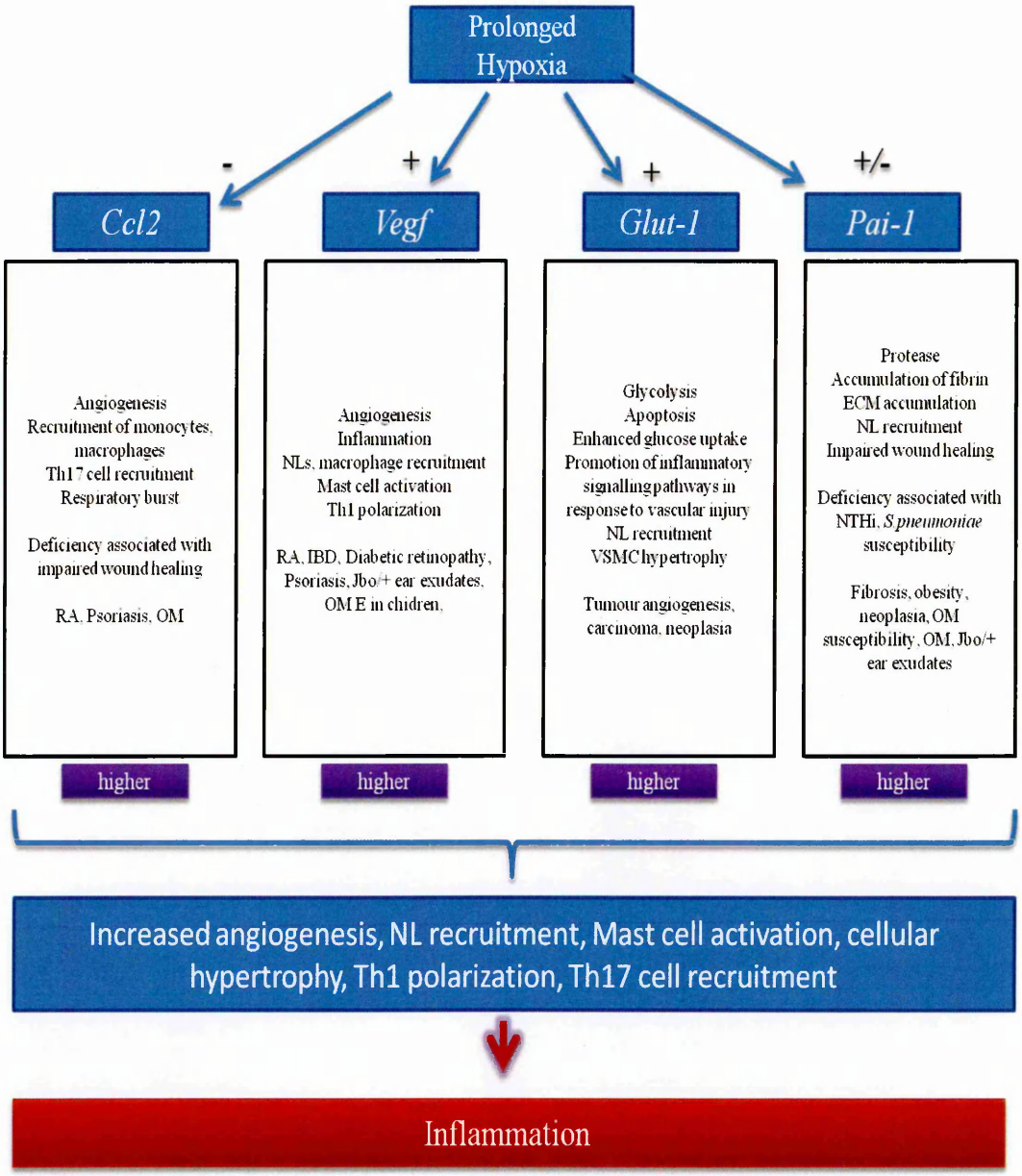


Figure 7.1- HIF signalling is dysregulated in *Jbo/+* BMDM

Potential loss of *Evi-1*^{A2288T} function as co-repressor of Smad3 resulted in induction of *Vegf, Glut-1* due to enhanced Smad3 activity. Higher levels of *Pai-1* and *Ccl2* despite a downregulation of expression under prolonged hypoxic condition were also observed. Higher levels of *Vegf, Glut-1, Ccl2,* and *Pai-1 in Jbo /+* BMDM compared to WT BMDM may result in enhanced angiogenesis, NL recruitment, mast cell activation, cellular hypertrophy and Th17 cell recruitment exacerbating inflammation.

6.3.6- Activated *Jbo/+* BMDM are LPS-hyper-responsive in normoxia but have attenuated responses in hypoxia

6.3.6.1- *Jbo/+* BMDM are hyper-responsive for *Vegf* in normoxia

Vegf expression was upregulated with 2 h and 24 h LPS treatment in a time-dependent manner in both WT and *Jbo/+* BMDM. Interestingly, LPS induced *Vegf* activation was 123% higher in *Jbo/+* BMDM (13 fold upregulation in WT, 29 fold upregulation in *Jbo/+*) at 24 h LPS activation in normoxia. This difference in *Vegf* expression levels was significant ($P < 0.05$). This trend of higher *Vegf* levels in *Jbo/+* was clearly apparent with 24 h LPS treatment in hypoxia as well but the difference was not significant. *Vegf* is also upregulated by just LPS under normoxic conditions via the SP-1 transcription factor (Sakuta et al, 2001). LPS can increase *Vegf* expression by increasing activity of NF-κB pathway as well because *Vegf* promoter has Iκβ binding site (Karin, 2006). LPS treatment can also increase *Vegf* expression by increasing Hif-1α expression (Blouin et al, 2004). LPS induced expression levels of *Vegf* was greater than Vegf induction by hypoxia alone. *Vegf* gene and protein expression has been found in the middle ear of LPS induced OME model (Jung et al, 1999). VEGF has also been detected in middle ear effusions from children with OME (Sekiyama et al, 2010). Injection of recombinant Vegf in rat middle is associated with fluid effusion and mucosal inflammation (Kim et al, 2005). These findings suggest that *Vegf* is produced in inflamed middle ear in response to LPS and hypoxia. Recently HIF-VEGF signalling pathway has also been shown to be upregulated in *Jbo/+* and *Jf/+* middle ear fluids (Cheeseman et al, 2011). VEGFR signalling inhibitors have also been found to suppress OM in *Jbo/+* implicating a role of *Vegf* in OM development (Cheeseman et al, 2011). My

results indicate that when induced with hypoxia or endotoxin, *Jbo/+* BMDM are prone to more *Vegf* production than WT. Increased *Vegf* levels in hypoxic or activated conditions can contribute to OM by recruiting more NLs, accumulating middle ear fluids and activating mast cells. *Vegf* can also polarise T cells to differentiate into pro-inflammatory Th1 cells by increasing IFN-γ production and decreasing Il-10 levels (Mauro et al, 2008; Mor et al, 2004).

6.3.6.2- *Jbo/+* BMDM have attenuated *Il-1β* levels in hypoxia and in activated hypoxic state

Il-1β is a prototypical inflammatory cytokine encoding gene. Il-1β is released by macrophages, fibroblasts, mast cells, DCs and NK cells and is involved in a range of inflammatory functions such as the upregulation of adhesion molecules, inflammatory cell infiltration, synthesis of NO, production of IL-6 and acute-phase proteins, apoptosis, angiogenesis, antibody formation, differentiation of T cells to pro-inflammatory Th17 cells etc (Dinarello, 2009). Mice deficient in *Il-1β* exhibit no spontaneous inflammation but instead display reduced systemic and local inflammation, reduced acute phase response but increased disease susceptibility to live pathogens (Dinarello, 2009). *Il-1β* is upregulated by LPS as well as by hypoxia (Hume et al, 2002; Locati et al, 2002; Perera et al, 1998; Scannell, 1996). Il-1β is implicated in chronic inflammation such as RA, IBD, osteoarthritis, diabetes, Alzheimer's disease etc (Dinarello, 2009). Higher *Il-1β* levels have been observed rat model of AOM as well as *Jbo/+* ear exudates (Cheeseman et al, 2011; Tong et al, 2008). Polymorphisms in *IL-1β* have been associated with COME/RAOM in humans (Rye et al, 2011a; Sale et al, 2008).

Il-1β levels were upregulated as expected with LPS activation of BMDM in a time dependent manner. LPS induced levels of *Il-1β* were comparable in both genotypes under normoxia conditions. Hypoxia enhanced the LPS induced upregulation of *Il-1β* levels. However, *Il-1β* levels were 30% lower ($P < 0.05$) in hypoxic activated *Jbo/+* BMDM at the short time-point of 2h but the levels reach WT levels on prolonged hypoxia. Although Smad3 is known to increase increase expression of Il-1β protein suggesting an autocrine loop between TGF-β pathway and Il-1β protein expression (Aoki et al, 2006), reduced *Il-1β* activation under hypoxia and LPS activation at 2 h can potentially be attributed to hypothetical loss of function of A2288T *Evi-1* as activator of AP-1 which acts to upregulate expression of pro-inflammatory genes including *Il-1β* (Tanaka et al, 1994). Reduced *Il-1β* levels in *Jbo/+* BMDM under hypoxic conditions can potentially result in attenuation of macrophage function and susceptibility to bacterial infections independently. Although, it is important to note here that *Jbo/+* mice develop OM in germ-free conditions as well.

6.3.6.3- *Jbo/+* BMDM has lower *Tnf-α* level in LPS activated BMDM in hypoxia

TNF-α is a pro-inflammatory cytokine which is involved in M1 polarization (along with IFNγ), apoptosis, cell proliferation and orchestration of pro-inflammatory cytokine response via activation of transcription factors such as NF-κBand AP-1 (Aggarwal, 2003; Chen & Goeddel, 2002; Parameswaran & Patial, 2010). TNF-α is produced by macrophages, mast cells, epithelial cells and stromal cells and is often associated with chronic inflammation such as atherosclerosis, IBD and arthritis (Clark, 2007; Parameswaran & Patial, 2010). Elevated TNF-α is also a primary cytokine

often associated with OM (Juhn et al, 2008; Skotnicka & Hassmann, 2000; Smirnova et al, 2002). Polymorphisms in the *TNF-α* gene are associated with AOM (TNFA-238G/G, 376 G/G) (Emonts et al, 2007a; Patel et al, 2006). In induced AOM models in rat and mice, increased TNF-α expression after NT*Hi* transtympanic inoculation has been observed (Catanzaro et al, 1991; Melhus & Ryan, 2000). These studies indicate a role of TNFα induction in AOM. Higher *Tnf-α* Levels are also observed in *Jbo/+* ear exudates (Cheeseman et al, 2011). However, persistent inflammation in induced OM in *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice has been associated with defective induction in *TNF-α* gene after infection (Leichtle et al, 2009; Leichtle et al, 2010). *TNF-α* deficient mice also display OM similar to *TLR2* and *MyD88* mutants with a reduced NT*Hi* phagocytosis by macrophages. In *Tnf-α*$^{-/-}$ mice there is diminished but prolonged NL recruitment in middle ear. Macrophages from *Tnf-α*$^{-/-}$ mice display attenuated bactericidal phagocytosis which indicates impairment of macrophage function. TNFα also plays a major role in apoptosis and as a delayed OM resolution in *Tnf-α*$^{-/-}$ deficient mice (Leichtle et al, 2009) it might indicate a role of TNF-α in apoptotic function and in recovery of OM.

Results from this work indicate that no difference was observed in *Tnf-α* gene levels between genotypes at basal level, hypoxia or LPS activation in normoxia. However, LPS induced *Tnf-α* expression in hypoxia was reduced in *Jbo/+* BMDM by 36% vs. treated WT BMDM at 2h ($P < 0.05$). This trend was maintained at 24h with a 23% reduction in *Jbo/+* but the difference between the genotypes was not significant. Tnf-α is known to decrease scavenger receptor gene and protein expression (Hsu et al, 1996), reduced Tnf-α levels in hypoxia and infection may predispose *Jbo/+* BMDM to foam cell formation and attenuate macrophage function.

6.3.6.4- *Jbo/+* BMDM are hyper-responsive to LPS for *Il-6* expression in normoxia

IL-6 is a multi-functional cytokine involved in acute phase response (Gabay, 2006). IL-6 protects T cell from apoptosis, promotes NL apoptosis and influences T cell polarization to inhibit T-regs and induce Th17 differentiation in presence of TGF-β (Bettelli et al, 2006; Ishihara & Hirano, 2002; Jones, 2005; Kishimoto, 2010). IL-6 is associated with RA, colitis, encephalomyelitis and development of inflammation owing to its role the transition from NL to macrophage inflammation (Alonzi et al, 1998; Hurst et al, 2001; Yamamoto et al, 2000). Il-6 levels are also upregulated in ear effusions from induced OM in mice (Barzilaia et al, 2000; Yellon et al, 1992). Upregulated Il-6 levels have also been reported in induced AOM in rats after NT*Hi* inoculation (Melhus & Ryan, 2000). Upregulated IL-6 protein is seen in humans in choleastoma tissue from patients with chronic OM (Kuczkowski et al, 2011). Polymorphism in *IL-6* at position 174 is associated with OM in humans (Emonts et al, 2007a; Patel et al, 2006).

IL-6 is upregulated with LPS in an NF-κB dependent manner (Begum et al, 2004; Yang et al). IL-6 levels in *Jbo/+* BMDM were relatively higher by 42% on LPS activation at 24 h normoxia ($P < 0.05$). A trend of higher IL-6 levels in *Jbo/+* was also present at 24 h LPS activation in hypoxia but the difference was not significant. Higher IL-6 level in normoxic LPS activated *Jbo/+* BMDM would potentially result in amplified inflammatory response, increased M1 activation and Th1 polarization to result in chronic inflammation.

These results reveal a dysregulation in HIF signalling but this regulation may be regulated by more than just Hif-1α. LPS results show that NF-κBmight have a role in HIF dysregulation observed in *Jbo/+*.

6.3.6.5- *Jbo/+* foamy BMDM produce lower *Nos-2* under hypoxia – implication for attenuated macrophage function

Jbo/+ ear exudates are characterized by presence of foamy macrophages (Cheeseman et al, 2011; Parkinson et al, 2006). Foamy macrophages are formed by uptake of ox-LDL which gives these macrophages a characteristic foamy appearance (Brown et al, 2000; Heinecke et al, 1991). Hypoxia is reported to promote foam cell formation and hypoxia in *Jbo/+* middle ear would be conducive to foam cell formation (Cheeseman et al, 2011; Hulten & Levin, 2009; Parkinson et al, 2006; Rydberg et al, 2004). The Ox-LDL concentration in *Jbo/+* ear exudates is approximately 50 pg/ml and is 245 fold greater than in *Jbo/+* or WT serum. To test if the presence of foamy macrophages,Ox-LDL, and with hypoxia might affect the inflammatory response in *Jbo/+* mice, oxysterol studies on *Junbo* BMDM were performed. Although no defect in expression of cholesterol efflux was evident as *Abca1* expression levels were comparable in both genotypes with oxysterol treatment in normoxia and hypoxia, anomalous *Nos-2* expression was observed in *Jbo/+* BMDM with oxysterol treatment in hypoxia.

Nos-2 is a gene that encodes for NO generating enzyme Nos-2 (Leone et al, 1991; Palmer et al, 1988). *Nos-2* is expressed by macrophages, monocytes, NLs, eosinophils, DCs, mast cells, NK cells, endothelial cells, epithelial cells, VSMCs and fibroblasts in response to activation by LPS, hypoxia or pro-inflammatory cytokines such as Tnf-α, Il-1β, Ifn-γ (Bogdan et al, 2000). *Nos-2* expression is upregulated by LPS via NF-κB(Heitmeier et al, 1998;

Song et al, 2004). Its expression is also upregulated with hypoxia (Angele et al, 1999; Blouin et al, 2004). Although no difference in *Nos-2* levels of biological significance was observed on LPS or hypoxic treatment of *Junbo* BMDM on its own or in combination; a significant genotypic difference in Nos-2 levels between genotypes was observed on oxysterol treatment of *Junbo* BMDM. *Nos-2* levels were 76% lower in *Jbo/+* BMDM on oxysterol treatment in hypoxia ($P < 0.05$) and a trend of lower *Nos-2* was also observed in normoxic oxysterol treatment although the difference was not significant. Nos-2 is essential for pathogen clearance, and plays an immunoregulatory role by affecting differentiation of Th1 cells and cytotoxic function of NK cells *Nos-2*$^{-/-}$ mice have been reported to be extremely susceptible to infections, bolstering the evidence for the role of Nos-2 in pathogen clearance. Lower NO might also make *Jbo/+* BMDM prone to bacterial infection under high Ox-LDL and hypoxic conditions.

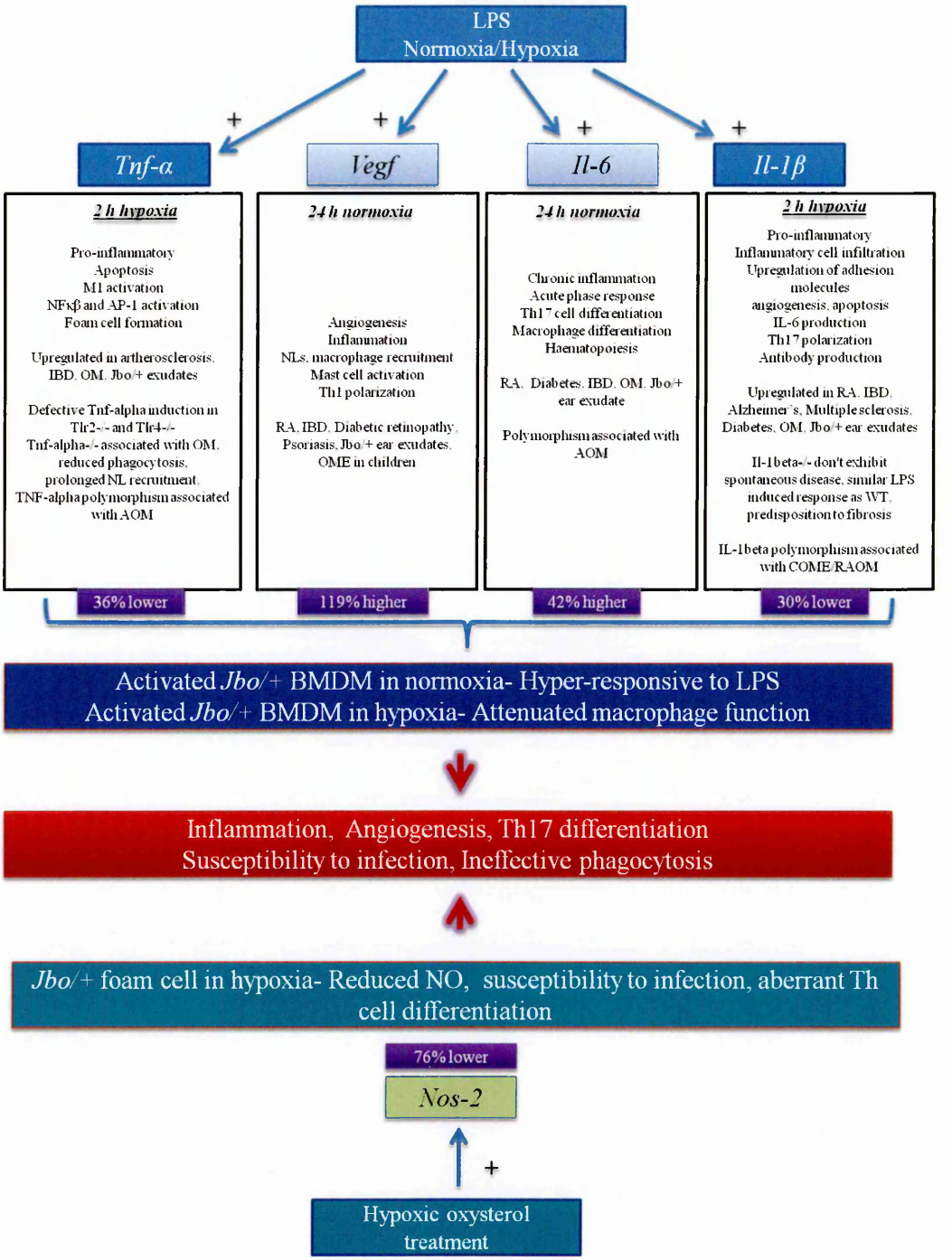


Figure 7.2-*Jbo/+* BMDM are LPS-hyper-responsive in normoxia but activated *Jbo/+* BMDM have attenuated macrophage function in hypoxia

Jbo/+ BMDM are hyper-responsive to LPS in normoxia as is evident by higher levels of pro-inflammatory *Vegf, Il-6* expression. However, under hypoxic conditions, activated *Jbo/+*BMDM possess attenuated macrophage function with reduced *Tnf-α* and *Il-1β* levels. Oxysterol treated *Jbo/+* BMDM are also hypo-responsive and have lower levels of *Nos-2.* These properties of *Jbo/+* BMDM make them prone to inflammation in both normoxia and hypoxia.

6.3.7- Resolution of inflammation in *Jbo/+*BMDM is impaired in hypoxic conditions

Hypoxia can either exacerbate inflammation or resolve inflammation as hypoxic macrophages express both pro-inflammatory M1 and an anti-inflammatory M2 markers (Rahat et al, 2011). However, resolution of inflammation is dependent on phagocytic activity of macrophages as well as well-timed suppression of pro-inflammatory mediators. An abnormality in either process can result in chronic inflammation. Pro-inflammatory gene expression can delay resolution of inflammation if this expression is prolonged or at a relatively higher level.

Hypoxic conditions are prevalent in inflamed middle ear in *Jbo/+* mice and results from this thesis indicate that under hypoxic conditions, *Jbo/+* BMDM have dysregulated resolution of inflammation as is evident from higher level of pro-inflammatory gene expression levels of *Il-6* (46% higher) and *Il-1β* (30% higher) ($P < 0.05$). Higher levels of Il-1β, Il-6 can pre-dispose hypoxic *Jbo/+* site to prolonged inflammation by prolonged release of acute phase proteins and downstream pro-inflammatory effectors and increasing inflammatory cell infiltration (Figure 6.3). Higher IL-6 and IL-1β levels are associated with differentiation of a subset of T cells known as Th17 cells which are associated with chronic inflammation and autoimmunity (Bettelli et al, 2006). This indicates that impairment of resolution in *Jbo/+* BMDM under hypoxic conditions can exacerbate inflammation and drive it towards chronicity.

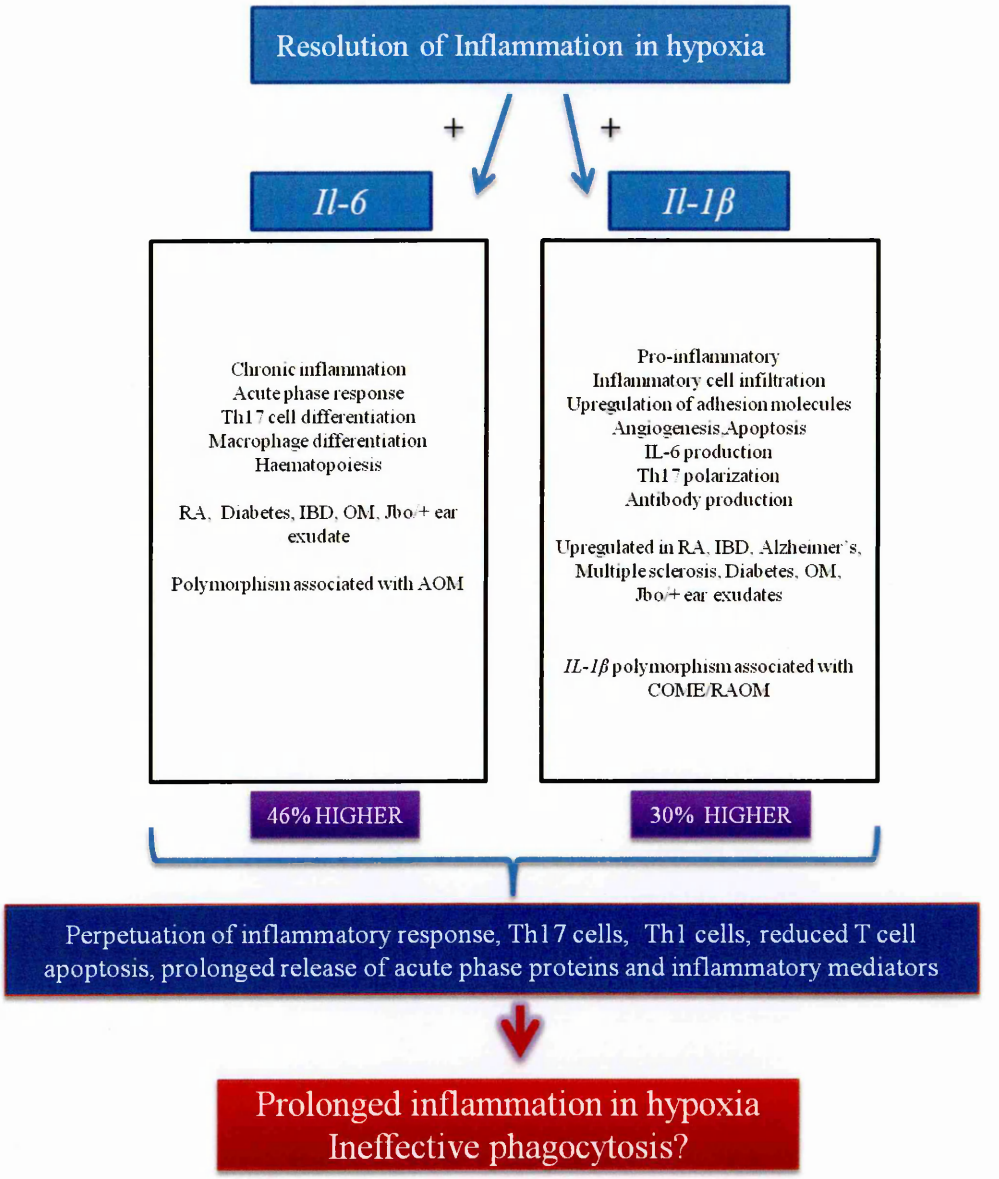


Figure 7.3-*Jbo/+* BMDM display impaired resolution of inflammation in hypoxia

LPS activation for 2 h in normoxia followed by LPS washout and subsequent hypoxic treatment for 24 h revealed that levels of pro-inflammatory genes were higher in *Jbo/+* than WT indicating a potential abrogated resolution of inflammation under hypoxic conditions.

6.3.8- Aberrant TGF-β signalling in *Jbo/+* BMDM in normoxia and hypoxia- indications of involvement of Smad and non-Smad dependent pathways

6.3.8.1- *Vegf* levels are differentially dysregulated with TGF-β treatment in normoxia and hypoxia

Angiogenesis is required for maintenance of any chronic inflammatory condition as VEGF acts through multiple signalling pathways to stimulate cell proliferation, cellular migration and recruitment (Teige et al, 2009; Walshe et al, 2009). Inflammatory cytokines often upregulate angiogenic mediators such as VEGF. Vascular changes have been linked to a number of chronic inflammatory diseases and increased *Vegf* levels have been observed in diseases such as RA, IBD and diabetic retinopathy (Angelo & Kurzrock, 2007). *Vegf* expression has been associated with increased recruitment of NLs, macrophages and increased mast cell activation (Angelo & Kurzrock, 2007; Canavese et al, 2010; Detmar et al, 1998).

Vegf expression pattern with TGF-β treatment was quite interesting as *Vegf* expression was upregulated by TGF-β treatment in normoxia and hypoxia. However, genotypic differences were observed only at 6 h time-point where *Vegf* expression was 30% lower in *Jbo/+* BMDM than WT ($P < 0.05$) in normoxia. Interestingly *Vegf* levels were comparable with further exposure to TGF-β and hypoxia. One possibility is that *Vegf* may be regulated by 2 different mechanisms (via pathways other than Smad3 and Hif-1α) in *Jbo/+* BMDM in presence of TGF-β, which may be affected by *Evi-1*^{A2288T} mutation.

6.3.8.2- *Jbo/+* BMDM has lower *Pai-1* levels under TGF-β treatment in normoxia

Pai-1 levels were lower in *Jbo/+* BMDM with 6 h TGF-β treatment in normoxia by 38% ($P < 0.05$). This is interesting as *Pai-1*is a hypoxia responsive gene (Liao et al, 2007) as well as a Smad3 responsive gene (Dennler et al, 1998b; Stroschein et al, 1999). *Pai-1* expression is induced by different signaling pathways as *Pai-1* promoter has various cis-regulatory sites such as SP1 site, AP-1 sites, HRE sites and Smad binding sites which indicates complex transcriptional regulation of this gene (Liao et al, 2007; Nagamine, 2008). It is possible that the *Evi-1*^{A2288T} mutation in *Jbo/+* regulates *Pai-1* gene expression through another novel pathway. In humans, polymorphisms in *PAI-1* gene have been linked to higher *PAI-1* levels and recurrent AOM (Emonts et al, 2007b). Higher *PAI-1* levels due to *PAI* 4G/4G genotype in humans and plasminogen deficient mice both display impaired wound healing rather than affect on OM susceptibility (Emonts et al, 2007b; Eriksson et al, 2006). Lower *Pai-1* levels in *Jbo/+* mice might not be the main reason behind OM but might predispose *Jbo/+* mice to bacterial infection under hypoxic conditions.

6.3.8.3- *Jbo/+ BMDM* has higher *Il-6* levels under TGF-β treatment in hypoxia

Increased levels of Il-6 (214%, $P < 0.05$) under prolonged conditions of TGF-β and hypoxia in *Jbo/+* BMDM can potentially predispose *Jbo/+* BMDM to inflammation due to increased levels of acute-phase proteins and induce differentiation of pro-inflammatory Th17 cells (Figure 6.4).

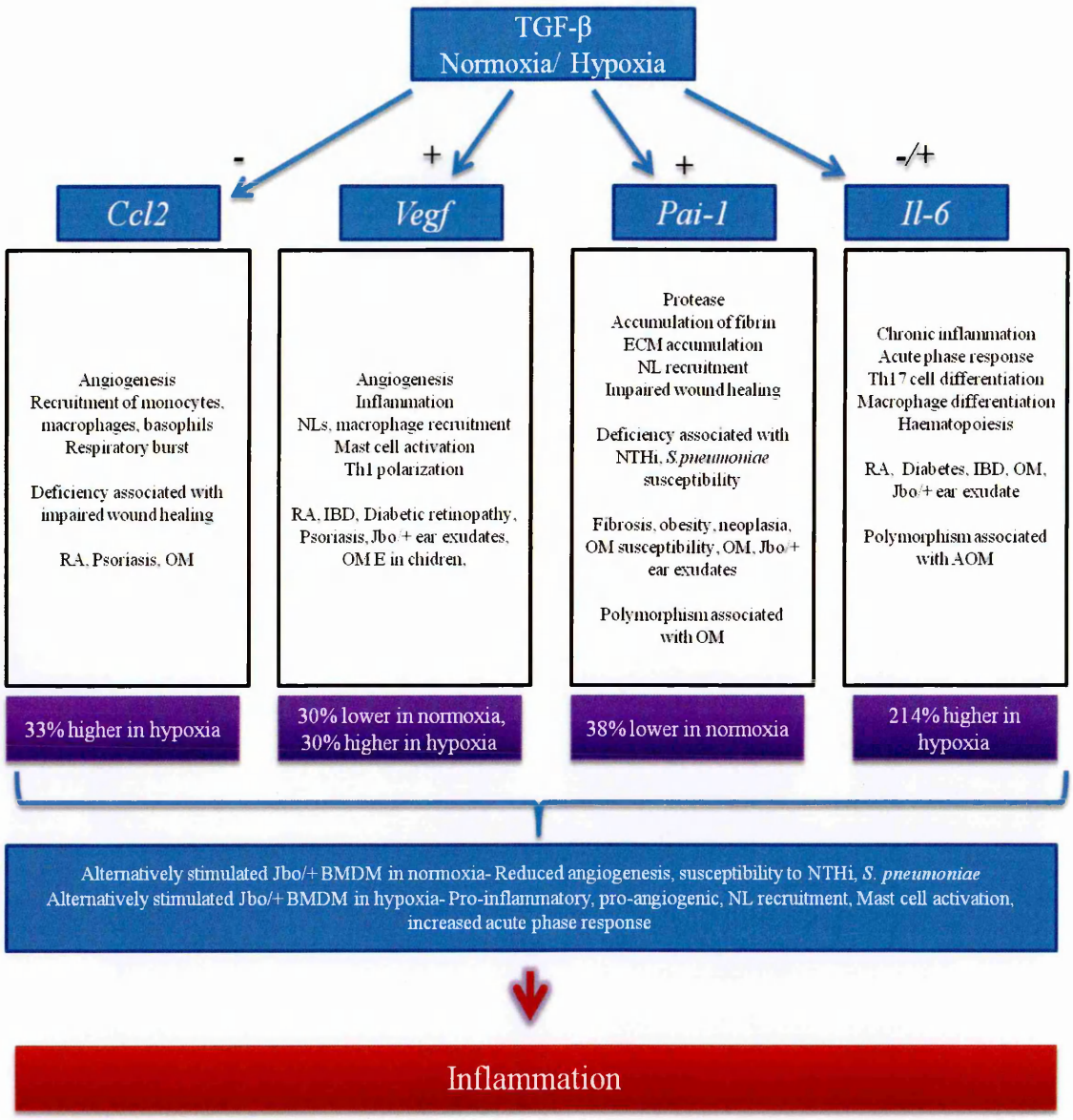


Figure 7.4-*Jbo/+* BMDM displays dysregulated TGF-β signalling in normoxia and hypoxia

TGF-β signalling is dysregulated in *Jbo/+* BMDM. Higher levels of pro-inflammatory *Il-6* in *Jbo/+* BMDM may predispose them to inflammation under conditions of hypoxia

6.3.8.4- Jbo/+ BMDM are predisposed to inflammation at basal level

Comparative protein expression studies in this work revealed that *Jbo/+* BMDM have upregulated levels of pro-inflammatory cytokines Il-23, C5a, Tnf-α, Trem-1, Il-17 as well as lymphocyte chemoattractants I-tac, Il-16 under resting condition (Figure 6.5). These cytokines induce chemotaxis of pro-inflammatory Th1 cells, eosinophil and monocytes. *Jbo/+* BMDM also possessed higher level of B cell chemoattractant Cxcl13 and proinflammatory tissue inflammation associated cytokine 1l-17. High levels of pro-inflammatory cytokines and low levels of anti-inflammatory cytokines Il-4, Il-2, Il-1ra may potentially make *Jbo/+* BMDM prone to inflammation and hyper-responsive to LPS in normoxic conditions. High Trem-1 levels may serve to amplify inflammatory responses in *Jbo/+* BMDM .

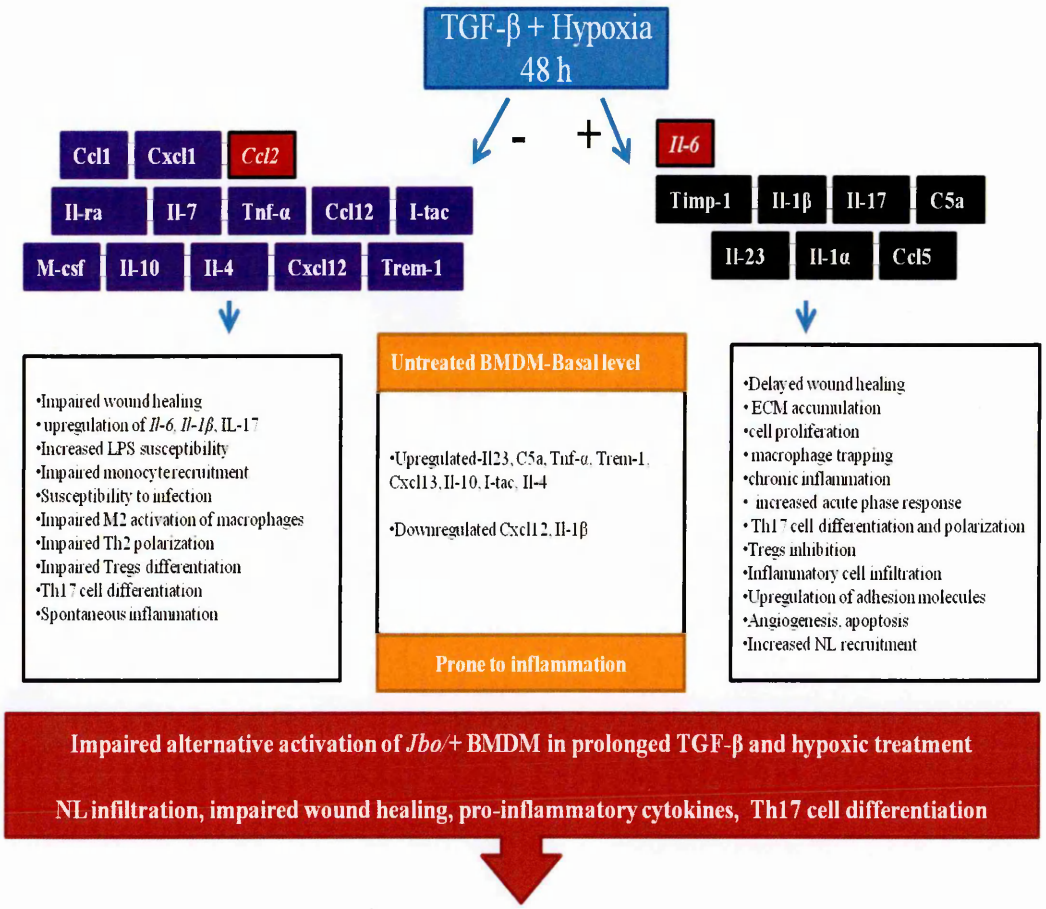


Figure 7.5-Differential cytokine expression in *Jbo/+* indicates predisposition to inflammation at both basal level and after 48 h hypoxia and TGF-β treatment

At basal level *Jbo/+* BMDM had upregulated levels of pro-inflammatory cytokines as well as anti-inflammatory cytokines

6.3.8.5- *Jbo/+* BMDM are potentially pro-inflammatory, promoting Th17 cell differentiation and dysregulated wound healing under conditions of prolonged hypoxia and TGF-β treatment

Ingestion of apoptotic of NLs is reported to stimulate the release of TGF-β which alternatively activates macrophages and primes them to resolve inflammation (Edwards et al, 2006; Martinez et al, 2009; Mosser & Zhang, 2008a). To mimic resolving inflammation in *Jbo/+* ears; TGF-β and hypoxia treatment of BMDM was performed.

Lower levels of key anti-inflammatory cytokines IL-1ra, IL-10, IL-2, Ccl2, and Ccl12 potentially predispose *Jbo/+mice* to impaired wound healing, spontaneous inflammation and infection susceptibility under conditions of prolonged hypoxia (48 H) in presence of TGF-β.

Jbo/+ BMDM also had higher levels pro-inflammatory Il-6, Il-23, Il-1β, Ccl5, Il-17, Il-16 and anaphylatoxin C5a in prolonged hypoxia (48 h) in presence of TGF-β treatment which would potentially result in increased levels of acute phase protein, increased respiratory burst, infiltration of NLs, eosinophils, monocytes, T cells as well as activation of macrophages, T cells, NLs and mast cells to release proinflammatory mediators.

Reduced levels of T cell and B cell growth factor Il-7, Mcsf, Ccl2, Cxcl13, Ccl12 and Th1 polarizing cytokine I-tac after treatment indicate a potential deficit in chemotaxis and differentiation of lymphocytes, macrophages, monocytes in prolonged hypoxic *Jbo/+* BMDM in presence of TGF-β which can prove detrimental for resolution of inflammation.

Interestingly *Jbo/+* BMDM released cytokines favourable for Th17 differentiation. Th17 cells are a subset of T cells which are associated with chronic inflammation (Littman & Rudensky, 2010; Liu et al, 2011).

Depending upon cytokines released in inflammatory microenvironment, CD4+ Th cells can differentiate into Th1, Th2, Th17 or Tregs. Tregs and Th17 cells have inverse roles in inflammation and their differentiation is dependent upon TGF-β and other cytokines. Tregs are immunosuppressive and are induced by activation of naïve T cells by IL-10 or TGF-β (Foxp3+). Tregs are important 'self check' T cells which are critical to control excessive inflammation and cytokines released by *Jbo/+* BMDM on prolonged TGF-β and hypoxia exposure describe an environment that potentially will inhibit Treg differentiation and facilitate Th17 differentiation to result in chronic inflammation and PMN infiltration in the middle ear. However, Il-6 and IL-1β are reported to suppress Tregs differentiation and induce Th17 differentiation.

Th17 cells are associated with autoimmune diseases such as EAE, IBD, psoriasis and RA and are formed by differentiation of naïve T cells by IL-6, TGF-β and their maintenance and differentiation is supported by IL-17, IL-23 and IL-1β. Il-2 and Il-4 are known to decrease Th17 differentiation.

Higher levels of Il-6, ll-1β, Il-17 in *Jbo/+* potentially may divert development of Foxp3+ Tregs towards Th17 lineage under conditions of prolonged TGF-β and hypoxia. Higher levels of Il-23 and lower level of Il-10, Il-2 and Il-4 can potentially maintain differentiation of Th17 cells leading to chronic inflammation.

6.4- Potential molecular pathways of disease development in *Jbo/+* mice

Data in this thesis has revealed that dysregulation of HIF signalling pathway and TGF-β signalling pathway exists in *Jbo/+* BMDM. Protein expression studies in *Jbo/+* BMDM reveal that they are potentially pre-disposed to inflammation at basal level due to higher levels of pro-inflammatory and NF-κBtarget proteins such as Il-23, Il-17, Tnf-α, I-tac, C5a, Trem-1 (Figure 6.5). Gene expression studies in this thesis have also revealed that *Jbo/+* BMDM are potentially hyper-responsive to LPS in normoxia with respect to *Il-6* and *Vegf* signaling. *Vegf* has been implicated in OM in *Jbo/+* mice previously (Cheeseman et al, 2011). *Jbo/+* BMDM also display pro-angiogenic property and impaired resolution of inflammation in hypoxia. However, the pathways behind OM susceptibility of *Jbo/+* need to be elucidated further. In this thesis, I hypothesized that because Evi-1 gene or protein expression is comparable in the 2 genotypes, ZF2 A2288T mutation in ZF2 of *Evi-1* potentially affects $Evi\text{-}1^{A2288T}$ function as a transcription factor and co-repressor.

A characterized function of Evi-1 is as a co-repressor of Smad3 by direct interaction via ZF1 and recruitment of CtBP (Izutsu et al, 2001; Kurokawa et al, 1998b). I hypothesized that loss of function of Evi-1 due to $Evi\text{-}1^{A2288T}$ mutation would result in increased activity of Smad3. Smad3 and other Smad transcription factors have low affinity with DNA and must interact with other transcription factors to form stable complexes with DNA (Mullen et al, 2011). My hypothesis was that transcriptional activity of Smad3 would be augmented under hypoxia as Smad3 is known to interact with Hif-1α in hypoxia to co-activate expression of *Vegf* (Sanchez-Elsner et al, 2001). My

hypothesis was validated as increased levels of Smad3 responsive and HIF responsive genes *Vegf, Glut-1* was observed in *Jbo/+* BMDM under conditions of prolonged hypoxia. *Vegf* induction was also higher in LPS activated and TGF-β and hypoxia treated BMDM. High levels of Smad3 responsive Timp-1 protein, Il-1β protein after 48 h treatment also validate the above outlined hypothesis as they are both Smad3 and HIF responsive proteins (Aoki et al, 2006; Bosco et al, 2008; Rius et al, 2008; Verrecchia et al, 2001). Lower levels of Ccl2 and Ccl12 (CCL2 structural and functional homologues in mice) after 48 h TGF-β and hypoxia treatment were also indicative of increased Smad3 transcriptional inhibitory activity under hypoxia due to potentially higher Smad3 levels (Feinberg et al, 2004).

Interestingly, high levels of Smad2 responsive *Il-6* gene were also observed in *Jbo/+* BMDM on prolonged TGF- β and hypoxia treatment. The role of Evi-1 as a repressor of Smad2 has been previously also described (Alliston et al, 2005). Smad2 is reported to increase *Il-6* expression in presence of TGF-β in DU145 cells (Park et al, 2003). Also, hypoxia is known to increase IL-6 expression (Eickelberg et al, 1999; Junn et al, 2000; Li et al, 2004). Thus in *Junbo* mice we may be observing an impaired inhibition of smad 2 by Evi-1 resulting in a more pro-inflammatory phenotype.

Other possible mechanisms via which $Evi\text{-}1^{A2288T}$ exerts its affects could be as follows. Evi-1 is also known to activate AP-1 activity and repress RUNX1 activity via its second zinc finger domain (Senyuk et al, 2007; Tanaka et al, 1994). AP-1 is a transcriptional factor which interacts with both HIF and TGF-β signalling as well as other signalling pathways. Lower levels of some AP-1 responsive genes such as Pai-1, Ccl2, Il-4 (Johnston et al, 2000; Schmidt-Weber et al, 2000; Wang et al, 1999) were seen in *Jbo/+* BMDM with TGF-β treatment in hypoxia. AP-1 is also known to activate Il-

10 transcription in Th2 cell (Rooney et al, 1994). This means that Evi-1 is involved in number of pathways and that *Evi-1*^{A2288T} mutation in *Jbo/+* might potentially dysregulate a number of pathways.

RUNX1 plays an essential role in differentiation and function of T cells. EVI-1 has been reported to interact with and inhibit RUNX1 activity via interaction with the second zinc finger domain (Senyuk et al, 2007). TGF-β is known to activate *RUNX1* expression (Wildey & Howe, 2009). TGF-β has also been shown to upregulate expression of master Th17 regulator RORγt and Foxp3 in T cells. Interactions between Runx1, RORγt and Foxp3 are known to regulate differentiation of Th17 and Tregs where Runx1 plays an important role in establishment of T cell lineage (Zhang et al, 2008; Zhou & Littman, 2009). Runx1 interacts with Retinoic acid receptor related orphan receptor gamma (RORγt) to induce Il-17 expression (Zhang et al, 2008). Runx1 can also interact with transcription factor Foxp3 to induce Treg differentiation in presence of TGF-β (Zhang et al, 2008). In such a scenario, the microenvironment plays a crucial role in directing T h17 or Treg differentiation. In presence of TGF-β CD4+ T cell express both RORγt and Foxp3 but Foxp3 antagonizes RORγt function and interacts with Runx1 to enable Treg differentiation. However, in presence of pro-inflammatory cytokines sych as Il-6 or Il-1β; Foxp3 function is inhibited and RORγt interacts with Runx1 to induce Th17 cell differentiation and upregulation of Il-17 (Adamson et al, 2009; Littman & Rudensky, 2010; Zhang et al, 2008; Zhou & Littman, 2009). Runx1 is crucial for Th17 cell development as transduction of *Runx1* induces Th17 cell differentiation while siRNA against *Runx1* antagonizes Th17 cell differentiation (Zhang et al, 2008).

It is possible that increased activity of Runx1 occurs in *Jbo/+* BMDM due to *Evi-1*^{A2288T} encoded protein would result in activation of Runx1. HIF-1α is known to enhance degradation of FOXP3 in 293T cells (Human Embryonic Kidney 293 cells) (Dang et al, 2011). RUNX1 is also known to interact with SMAD3 (Jakubowiak et al, 2000). Results from this thesis reveal that prolonged hypoxia and TGF-β treatment resulted in upregulated Il-6 expression. It is possible that under conditions of hypoxia, upregulated Il-6, Il-1β and Runx1 activation, Th17 differentiation is favoured resulting in chronic inflammation. Interestingly, data from this thesis also points that under prolonged hypoxia and TGF-β treatment, cytokines favourable for Th17 differentiation are secreted. Interestingly, impaired repression of Runx1 by *Evi-1*^{A2288T} encoded protein may also contribute to lower *Vegf* levels observed with TGF-β treatment in normoxia as Runx1 is known to act as a repressor for *Vegf* (Ter Elst et al, 2011). Runx1 is also known to downregulate Il-4 expression (Naoe et al, 2007). Interestingly lower levels of Il-4 were also observed in *Jbo/+* BMDM along with higher levels of Il-17 after 48 h TGF-β and hypoxia treatment.

Evi-1 is also known to repress PU.1 function via ZF1 and by disrupting PU.1 and c-Jun binding (Laricchia-Robbio et al, 2009). PU.1 is reported to control lymphopoeisis and myelopoeisis and high PU.1 level is known to favour NL and macrophage maturation. Impairment of 'PU.1 repressive' activity of *Evi-1*^{A2288T} would result in increased PU.1 activation which can potentially account for higher levels of Il-1β (Marecki et al, 2001)and lower levels of Il-4 and Il-10 (Chang et al, 2005) as is observed in *Jbo/+* BMDM after 48 h TGF-β and hypoxia treatment.

Recently, it has also been reported that Evi-1 represses Phosphatase and tensin homologue on chromosome 10 (*Pten*) transcription via first zinc finger domain and recruiting PcG proteins (Yoshimi et al, 2011). Pten acts as a negative regulator of PI3k/Akt/mTOR pathway which Evi-1 activates via Pten repression (Yoshimi et al, 2011). Hypothetically, Evi-1 loss of function would result in Pten overexpression and repression of PI3k/AKt pathway as PI3k/Akt/mTOR pathway is activated by growth factors. As Pten is known to activate *I-tac* promoter (Rani et al, 2002) and downregulate Il-1β expression (Wang et al, 2008; Yum et al, 2001), impaired *Evi-1*^{A2288T} repression of Pten may potentially explain decreased levels of I-tac and increased levels of Il-1β after 48 h TGF-β and hypoxia treatment. PI3k/Akt signalling pathway is known to induce expression of M-csf, *Trem-1*, Tnf-α, Il-1ra, Il-10 (Chen et al, 2008; Learn et al, 2001; Mandal et al, 2009; Pulendran, 2004). Abrogated PI3k/Akt signalling due to potential Pten overexpression due to impaired repression by *Evi-1*^{A2288T} encoded protein could contribute to decreased levels of these cytokines observed after 48 h TGF-β and hypoxia treatment. However, it is important to note that Evi-1 represses Pten in only cells with high expression of Evi-1. Although BMDM express high levels of Evi-1 but low levels of Evi-1 gene are expressed and additional experiments would need to be performed to check if Pten repressive function of Evi- holds true for BMDM.

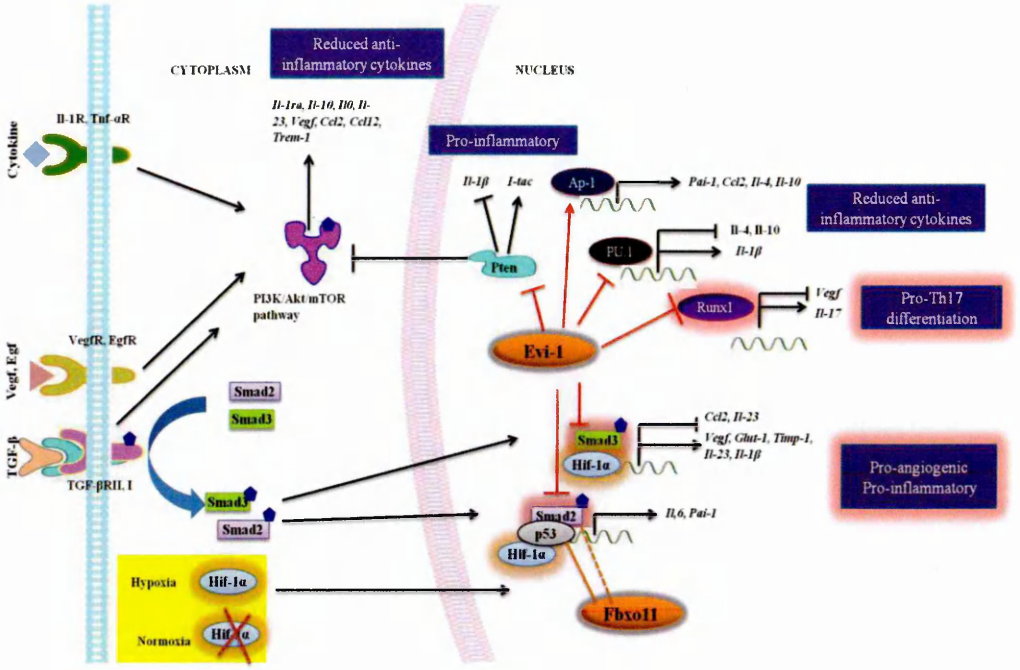


Figure 7.6-Potential pathway for OM development in *Jbo/+* and *Jf/+* mice

This figure illustrates putative pathways that maybe involved in OM pathogenesis in *Jbo/+* and *Jf/+* mice. Evi-1 is a co-repressor of Smad3 and Smad2 via ZF1. Evi-1 also represses transcription of transcription activator PU.1 and PI3K inhibitor Pten via ZF1. Evi-1 uses ZF2 to repress activity of Runx1 and activates AP-1 activity. A2288T mutation in *Evi-1* ZF2 coding region does not affect its expression in genotypes but may affect its binding and its function as a co-repressor or transcription activator. Similarly Fbxo11 is involved in stabilization of p53 which is required for TGF-β signalling via Smad2. *Fbxo11* expression level in both genotypes maybe equal but the *Fbxo11* protein in *Jf/+* is mutated and it is likely that the that the function of *Fbxo11* is perturbed in *Jf/+* mice. TGF-β triggers signalling via TGF-βRs which leads to phosphorylation of Smad2 and Smad3 and their subsequent transport to the nucleus where they interact with co-factors to modulate gene expression of their down-stream targets. Hypoxia is a key feature of inflammation in middle ear of *Jbo/+* and *Jf/+* mice. Hypoxia leads to stabilization of Hif-1α which interacts with both Smad2 and Smad3 in hypoxia to modulate gene expression. Prior evidence and results from this thesis indicate a dysregulation in HIF signalling as well as TGF-β signalling in *Jbo/+* and *Jf/+* mice which points to a common pathway for OM pathogenesis in both *Jf/+* and *Jbo/+* mice. However, the phenotypic difference in OM and its severity between the two models may be due to involvement of other factors in *Jbo/+* mice. TGF-β can also induce signalling via other pathways such as PI3K, Runx1, AP-1 which are all known to interact with Evi-1 and their activity maybe perturbed by *Evi-1*^{A2288T} mutation in *Jbo/+* mice.

6.5- Future work

Further studies need to be performed in *Jf/+* and *Jbo/+* to elucidate OM pathogenesis.

Data from this thesis reveals that *Jbo/+* BMDM are hyper-responsive to LPS and display attenuated resolution of inflammation in hypoxia. One of the first things to check next would be if clearance of apoptotic NLs is perturbed in *Jbo/+* BMDM under hypoxia using a simple protocol described by (Michlewska et al, 2009). It is plausible that pathogen clearance by *Jbo/+* BMDM also may also be affected under prolonged hypoxia as is indicated by reduced levels of *Tnf-α* and *Il-1β* levels in *Jbo/+* BMDM after 2 h LPS and hypoxia treatment. A bacterial challenge would be useful in confirming the finding. Comparison of cytokines after LPS activation and prolonged hypoxia between the genotypes using proteome profiler array would also indicate other cytokines whose expression is dysregulated between the genotypes and would confirm the RNA result.

To further elucidate the pathways that may be involved in OM due to *Evi-1*^{A2288T} mutation in *Jbo/+* mice, experiments with Smad3 and Runx1 overexpression in transgenic mice can be performed. Co-immunoprecepitation studies to study effect of *Evi-1*^{A2288T} mutation on the interactions of encoded protein with Runx1, PU.1, Smad3, Smad2 and AP-1 would also shed more light to the pathways involved in OM development in *Jbo/+* mice. Another thing to check would be the levels of *Pten* expression via RT-qPCR between WT and *Jbo/+* BMDM which would indicate whether Evi-1 repression of *Pten* is abrogated in *Jbo/+* BMDM.

Studies performed in this thesis also provide a model which can be replicated to study OM cellular processes in humans using peripheral monocytes to further confirm the findings and, highlight the role of hypoxia

in exacerbating inflammation in *Jbo/+* BMDM. It would be interesting to observe if induction of arthritis (another inflammatory disease with dysregulated HIF signalling) shows that *Jbo/+* BMDM are susceptible to a more aggressive disease.

Vegf, Glut-1, Il-6 were the key genes which were upregulated in *Jbo/+* under hypoxia and it would be interesting to observe if their overexpression in mice results in a phenotype similar to OM in *Jbo/+*. *Il-6* and *Il-1β* were higher in *Jbo/+* BMDM in resolution of inflammation study in hypoxia. Ultimately, the results from these studies and TGF-β studies can be potentially applied in a pharmacological and medical setting. At present OM is medically managed with antibiotics which are not very effective for OME and chronic OM (Browning et al, 2010; Griffin et al, 2006). Surgical intervention is the only way of treatment of chronic OM, in fact, each year 30,000 surgical procedures are carried out in UK alone to treat OM (Sood & Waddell, 2007). Recently, Vegf receptor inhibitors have been reported to significantly reduce OM severity in *Jbo/+* mice (Cheeseman et al, 2011). *Vegf* was reported to be upregulated in *Jbo/+* under hypoxia and LPS activation in normoxia in this thesis. In such a scenario, it is important to check if addition of Il-6 receptor inhibitor such as tocilizumab or Il-1β inhibitor Canakinumab or exogenous Il-ra (Anakira) and IL-10 can moderate OM severity in *Jbo/+* as expression of these markers was dysregulated in data presented in this thesis.

Data from this thesis also strongly hints towards a predisposition of *Jbo/+* BMDM in promoting differentiation of Th17 cell lineage in hypoxia and in presence of TGF-β. TGF-β is synthesized during apoptosis and potential induction of pro-inflammatory Th17 cells by *Jbo/+* BMDM in hypoxia *in vivo* might lead to chronic inflammation. Studies on Jbo /+ T cells would

shed more light on interactions between innate and cell mediated immunity in development of chronic OM in *Jbo* /+ mice.

Last but not the least, it is important to repeat studies performed on *Jbo*/+ BMDM in *Jf*/+ BMDM for more time-points to further understand OM pathogenesis in *Jf*/+ mice.

6.7- Contribution to the field

- Previously it has been reported that inflamed middle ear in *Jbo*/+ is hypoxic by our group (Cheeseman et al, 2011). Data from this independently confirmed the finding.
- This thesis describes a successful model for studying *in vitro* cellular processes in OM in *Jbo*/+ and *Jf*/+ which can be applied to other mice models.
- Protein expression data reveals that *Jbo*/+ BMDM are pro-inflammatory at basal level with higher levels of inflammatory cytokines such as Il-23, Trem-1, and C5a.
- *Evi-1* gene and protein expression is not affected due to $Evi\text{-}1^{A2288T}$ mutation in *Jbo*/+ BMDM rather the mutation is likely to be a loss of function mutation as its activity as a Smad3 co-repressor is perturbed.
- My results from this thesis indicate a pro-angiogenic nature of *Jbo*/+ BMDM in hypoxia despite attenuation of phagocytic function of *Jbo*/+ BMDM in hypoxia.
- Results indicate for the first time that *Jbo*/+ BMDM are hyper-responsive to LPS but possess impaired resolution of inflammation in hypoxia.

- Data suggests a of a loss of function of *Evi-1* and *Fbxo11* in *Jbo/+* and *Jf/+* BMDM respectively via perturbations in HIF-TGF-β signalling and TGF-β signalling pathways respectively.
- Data indicates a role of cytokines associated with Th17 differentiation in OM pathogenesis.

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